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(54) Title: **TRANSCRIPTIONAL REGULATION OF GENES ENCODING VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS**

BamHI
XhoI
HindIII
EcoRI
BamHI

-16.8kb      +1      +4.7kb

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-780 CCTCCCTGCC CTGGCCCTAA GATATCTTG GCTGGAAGCT CTGCTCTGAA AAGGGCCATG GCCAACTTT CACTAGGGCT CTGCTTGGG GAGCAGATG
-680 GACAAAAGCC TTCTTGGGGC TAGGCAGGTC ACTTCAAACT TGGAGCGGCC AAATATTTTC GGAATAGCG GGAATGCTGG CGAACTGGGC AAGTGGCTTT
-580 TCTGATTAA AGCAACCAGA TTCAGCTTTT TAAACTACAA TTATACTGGC CAACAAATAT ACCCTATATC AAAAACCAAA ACTACTGGCA GGAGTGGCTG
-480 CCAGCTTGGC ACCCGGCATA CTGGGCTGAG TATCGGCTTC TCCTTGTGGC CTCGAACTG CTGCAGATTC TCGGCCACTT CAGACCGCGC CGATGGCGAA
-380 GAGGCTCTGC CACTTTGACG CGCTGTGTGA GGGAGGGGTG CTCTTCGCAG CGCTCTGGT GATGCTCCCC AAATTTGGGG GACCGGCAAG CGATTAAATC
-280 TTGGAGTTGC TCAGCGCCCG TTACCGAGTA CTTTATATTT ACACAGAA CAAAGTTGTT GCTCTGGGAT GTTCTCTCTT GGGGACTTGG GGGCCGAGCG
-180 CAGTCAGATT GTGTCGGGAA ATGGCGAGAT GTAAATGGCC TGCGGAGCT GGAGATCCCG GCGGGCTACC CGGCTGAGGG GCGGGGCAAG CGGCACGGCA
-80 GAGGCTCTGC TCGGGCTGGG CGCGGCTGGC CATGGCGGCG CTTCGGGCT CTAGAGTTTC GGCTCCAGCT CCCACCTGCC ACTGAGTCCC GGAACCCCGG
+21 GAGAGCGGTC AGTGTGGGT CCGTGGTTTT CCGTGGCTGC GCGCGGGCAT CACTTGGCGG CGCAGAAAG TCCGTCTGGC AGCCTGGATA TCGTCTCTTA
+121 CCGGCAACCC GAGAGCGGCG TCGAGCGGCG GGTGGCGCGC CGGGCTCCCT AGCCCTGTGC GCTCAACTGT CCGCGGTCGC GGGGTGCGCG GAGTTCCACC
+221 TCGCGGCTTC CTTCTCTAGA CAGCGCGTGG GAGAAGAAC CGGCTCCCGA GTTCTGGCCA TTGGCCCGG CTCGAGGTGC AGGATGCACA GCAGGCTGCT
+321 GCTGGCGGTC GCGCTGTGGC TCTGGCTGGA GACCGCGGCC GCGCTGTGGG gtgaaggagcc cactctggyag gaggaaggca gacaggtcogy gtgagggcgy
+421 agaggaactg aaagccagat ctaactcgga atcgtagagc tggagagttg gacaggactt gacatttt (SEQ ID NO: 7)
  
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L A V A L W L C V E T R A A S V (SEQ ID NO: 15)

## (57) Abstract

The invention features substantially pure DNA containing an endothelial cell-specific promoter sequence capable of directing endothelial cell-specific transcription of a polypeptide-encoding sequence or an antisense template to which it is operably linked. The invention also features methods for identifying compounds which inhibit or enhance endothelial cell growth.

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- 1 -

TRANSCRIPTIONAL REGULATION OF GENES ENCODING VASCULAR  
ENDOTHELIAL GROWTH FACTOR RECEPTORS

Background of the Invention

5           This invention relates to endothelial cell-specific gene transcription and transcriptional regulation by TNF- $\alpha$ .

          Vascular endothelial growth factor (VEGF) is a potent and specific endothelial cell mitogen (Connolly et al., 1989, J. Clin. Invest. 84:1470-1478; Leung et al., 1989, Science 246:1306-1309). Through interactions with its receptors, Kinase-insert Domain-containing Receptor/fetal liver kinase-1 (KDR/flk-1) and flt1, VEGF plays critical roles in growth and maintenance of vascular endothelial cells and in the development of new blood vessels in physiologic and pathologic states (Aiello et al., 1994, New Engl. J. Med. 331:1480-1487; Shweiki et al., 1992, Nature 359:843-845; Berkman et al., 1993, J. Clin. Invest. 91:153-159). The patterns of embryonic expression of VEGF suggest that it is crucial for differentiation of endothelial cells from hemangioblasts and for development of blood vessels at all stages of growth (Jakeman et al., 1993, Endocrinology 133:848-859; Breier et al., 1992, Development 114:521-532). Among many potentially angiogenic factors, VEGF is the only one with patterns of expression, secretion, and activity that suggest a specific angiogenic function in normal development (Klagsbrun et al., 1993, Current Biology 3:699-702).

30           High-affinity receptors for VEGF are found only on endothelial cells, and VEGF binding has been demonstrated on macro- and microvascular endothelial cells and in quiescent and proliferating endothelial cells (Jakeman et al., 1993, Endocrinology 133:848-859; Jakeman et al.,

- 2 -

1992, Clin. Invest. 89:244-253). The tyrosine kinases KDR/flk-1 and flt1 have been identified as candidate VEGF receptors by affinity cross-linking and competition-binding assays (de Vries et al., 1992, Science 255:989-991; Millauer et al., 1993, Cell 72:835-846; Terman et al., 1992, Biochem. Biophys. Res. Commun. 187:1579-1586). These two receptor tyrosine kinases contain seven similar extracellular immunoglobulin domains and a conserved intracellular tyrosine kinase domain interrupted by a kinase insert (de Vries et al., 1992, Science 255:989-991; Matthews et al., 1991, Proc. Natl. Acad. Sci. U.S.A 88:9026-9030; Terman et al., 1991, Oncogene 6:1677-1683); they are expressed specifically by endothelial cells in vivo (Millauer et al., 1993, Cell 72:835-846; Peters et al., 1993, Proc. Natl. Acad. Sci. USA 90:8915-8919; Quinn et al., 1993, Proc. Natl. Acad. Sci. USA 90:7533-7537; Yamaguchi et al., 1993, Development 118:489-498). In situ hybridization in the developing mouse has demonstrated that KDR/flk-1 is expressed in endothelial cells at all stages of development, as well as in the blood islands in which endothelial cell precursors first appear (Millauer et al., 1993, Cell 72:835-846). KDR/flk-1 is a marker for endothelial cell precursors at their earliest stages of development (Yamaguchi et al., 1993, Development 118:489-498).

The vascular endothelium is critical for physiologic responses including thrombosis and thrombolysis, lymphocyte and macrophage homing, modulation of the immune response, and regulation of vascular tone. The endothelium is also intimately involved in the pathogenesis of vascular diseases such as atherosclerosis (Ross, 1993, Nature 362:801-809). Although a number of genes expressed in the endothelium have been characterized (Collins et al., 1991, J. Biol.

- 3 -

Chem. 266:2466-2473; Iademarco et al., 1992, J. Biol. Chem. 267:16323-16329; Jahroudi et al., 1994, Mol. Cell. Biol. 14:999-1008; Lee et al., 1990, J. Biol. Chem. 265:10446-10450), expression of these genes is either not limited to vascular endothelium (e.g., the genes encoding von Willebrand factor, endothelin-1, vascular cell adhesion molecule-1), or is restricted to specific subpopulations of endothelial cells (e.g., the gene for endothelial-leukocyte adhesion molecule-1).

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#### Summary of the Invention

The invention features substantially pure DNA, i.e., a promoter sequence, which regulates endothelial cell-specific transcription of a polypeptide-encoding sequence to which it is operably linked. The DNA of the invention contains a sequence substantially identical to nucleotides -225 to -164 of the KDR/flk-1 promoter, i.e., 5' TTGTTGCTCTGGGATGTTCTCTCCTGGGCGACTTGGGGCCCAGCGCAGTCCAGT TGTGTGGG 3' (SEQ ID NO:1). By "substantially identical" is meant at least 80% identical to a reference DNA sequence, that is, up to 20% of the basepairs of the reference DNA sequence can be replaced with an alternative basepair (e.g., G-C replaced with A-T, T-A, or C-G), provided that the transcription-promoting activity of the altered sequence is the same or greater than that of the reference sequence. The DNA may also include a sequence substantially identical to nucleotides -95 to -77 of the KDR/flk-1 promoter, i.e., 5' GCTGGCCGCACGGGAGAGC 3' (SEQ ID NO:2), a sequence substantially identical to nucleotides -95 to -60 of the KDR/flk-1 promoter, i.e., 5' GCTGGCCGCACGGGAGAGCCCCTCCTCCGC CCCGGC 3' (SEQ ID NO:3), a sequence substantially identical to nucleotides +105 to +127 of the KDR/flk-1 promoter, i.e., 5' GGATATCCTCTCCTACCGGCAC 3' (SEQ ID

- 4 -

NO:4), or a combination thereof. Preferably, the 5' to 3' orientation of sequences is SEQ ID NO:1; SEQ ID NO:2 or SEQ ID NO:3; and SEQ ID NO:4. However, any orientation of these sequences which promotes endothelial cell-specific transcription is within the invention. The DNA may include a nonspecific sequence between any two of the defined sequences, and/or at either or both ends. Preferably, this nonspecific (i.e., sequence other than SEQ ID NO:1-4 will constitute no more than 80% of the entire promoter sequence. Most preferably, it is substantially identical to the sequence shown in Table 1 (SEQ ID NO:5) or Table 2 (SEQ ID NO:6).

A "substantially pure DNA," as used herein, refers to a DNA which has been purified from the sequences which flank it in a naturally occurring state, i.e., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in the genome in which it naturally occurs.

A substantially pure DNA containing a sequence substantially identical to nucleotides -225 to +268 of the KDR/flk-1 promoter (SEQ ID NO:5; Table 1) or nucleotides -225 to +127 of the KDR/flk-1 promoter (SEQ ID NO:6; Table 2) and which regulates endothelial cell-specific transcription of a polypeptide-encoding sequence or antisense template to which it is operably linked is also within the invention.

TABLE 1: -225 to +268

TTGTTGCTCTGGGATGTTCTCTCCTGGGCGACTTGGGGCCCAGCGCAGTCCAGTTGT  
 GTGGGGAAATGGGGAGATGTAAATGGGCTTGGGGAGCTGGAGATCCCCGCCGGGTAC  
 CCGGGTGAGGGGCGGGGCTGGCCGCACGGGAGAGCCCTCCTCCGCCCCGGCCCCGC  
 CCGCATGGCCCCGCTCCGCGCTCTAGAGTTTCGGCTCCAGCTCCCACTTGCCTG  
 GAGTCCCGGGACCCCGGAGAGCGGTCAAGTGTGTGGTCCGCTGCGTTTCTCTGCCTG  
 CGCCGGGCATCACTTGCGCGCCGAGAAAGTCCGTCTGGCAGCCTGGATATCCTCTC  
 CTACCGGCACCCGAGACGCCCCCTGCAGCCGCGGTGGCGCCCGGGCTCCCTAGCC  
 CTGTGCGCTCAACTGTCCTGCGCTGCGGGGTGCCGCGAGTTCCACCTCCGCGCCTCC  
 TTCTCTAGACAGGCGCTGGGAGAAAGAACCGGCTCCC (SEQ ID NO:5)

- 5 -

TABLE 2: -225 to +127

TTGTTGCTCTGGGATGTTCTCTCCTGGGCGACTTGGGGCCCAGCGCAGTCCAGTTGT  
 GTGGGGAAATGGGGAGATGTAAATGGGCTTGGGGAGCTGGAGATCCCCGCCGGGTAC  
 CCGGGTGAGGGGCGGGGCTGGCCGCACGGGAGAGCCCCCTCCTCCGCCCCGGCCCCGC  
 5 CCGCATGGCCCCGCTCCGCGCTCTAGAGTTTCGGCTCCAGCTCCCACCCTGCACT  
 GAGTCCCGGGACCCCGGGAGAGCGGTCAGTGTGTGGTCGCTGCGTTTCCTCTGCCTG  
 CGCCGGGCATCACTTGCGCGCCGCAGAAAGTCCGTCTGGCAGCCTGGATATCCTCTC  
 CTACCGGCAC (SEQ ID NO:6)

The DNA of the invention may be operably linked  
 10 to, and functions to regulate endothelial cell-specific  
 transcription of, a sequence encoding a polypeptide that  
 is not KDR/flk-1. Examples of such polypeptides include  
 tissue plasminogen activator (tPA), p21 cell cycle  
 inhibitor, and nitric oxide synthase. By "operably  
 15 linked" is meant able to promote transcription of an mRNA  
 corresponding to a polypeptide-encoding or antisense  
 template located downstream on the same DNA strand.

The invention also includes a vector containing  
 the DNA of the invention, a method of directing  
 20 endothelial cell-specific expression of a polypeptide by  
 introducing the vector into an endothelial cell, and an  
 endothelial cell containing the vector.

The vector of the invention can be used for gene  
 therapy, such as a method of inhibiting arteriosclerosis  
 25 in an animal involving contacting an artery of the animal  
 with the vector of the invention which directs the  
 production of a polypeptide capable of reducing or  
 preventing the development of arteriosclerosis, e.g., a  
 polypeptide which reduces proliferation of smooth muscle  
 30 cells, e.g., interferon- $\gamma$  or atrial natriuretic  
 polypeptide.

The invention also includes compositions and  
 methods of carrying out antisense therapy. For example,  
 the invention includes a substantially pure DNA with a  
 35 sequence substantially identical to SEQ ID NO:1 which  
 regulates endothelial cell-specific transcription of an  
 antisense template to which it is operably linked, e.g.,

- 6 -

an antisense template the transcription product of which prevents translation of mRNA into an endothelial cell polypeptide. By the term "antisense template" is meant a DNA which is transcribed into an RNA which hybridizes to mRNA. Preferably, the endothelial cell polypeptide is KDR/flk-1. For example, the antisense RNA transcript which binds to and thereby prevents or reduces translation of an mRNA encoding KDR/flk-1, a protein involved in angiogenesis, can be used to treat cancer by contacting a tumor site in an animal with the DNA of the invention to reduce or prevent angiogenesis at the tumor site.

Translation of other endothelial cell polypeptides may also be reduced or prevented in this manner. For example, translation of cell cycle proteins, coagulation factors, e.g., von Willebrand factor, and endothelial cell adhesion factors, e.g., intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) may be reduced or prevented.

The invention also features a method of measuring the ability of a candidate compound to modulate TNF- $\alpha$  downregulation VEGF receptor (e.g., KDR/flk-1 or flt1) gene expression. In this method, a cell containing the promoter of a VEGF receptor gene operably linked to a reporter gene is cultured in the presence of TNF- $\alpha$  and the candidate compound. The level of expression of the reporter gene is then determined as a measure of the ability of the candidate compound to modulate TNF- $\alpha$  downregulation of VEGF receptor gene expression.

Another method included in the invention involves measuring the ability of a candidate compound to modulate TNF- $\alpha$  inhibition of VEGF-induced endothelial cell proliferation. In this method, an endothelial cell is cultured in the presence of TNF- $\alpha$ , VEGF, and the candidate compound. The level of endothelial cell growth



- 7 -

is determined (e.g., by measurement of uptake of [methyl-<sup>3</sup>H]thymidine) as a measure of the ability of the candidate compound to modulate TNF- $\alpha$  inhibition of VEGF-induced endothelial cell proliferation.

5           The invention also features a method of inhibiting angiogenesis in a patient involving administering to the patient a non-TNF- $\alpha$  compound which activates the TNF- $\alpha$  pathway of downregulating VEGF receptor (e.g., KDR/flk-1 or flt1) gene expression in an endothelial cell.

10           An additional method of the invention for inhibiting angiogenesis in a patient involves administering to the patient a polypeptide which inhibits VEGF receptor (e.g., KDR/flk-1 or flt1) gene expression in an endothelial cell by binding to the TNF- $\alpha$ -responsive  
15 element in the promoter of the VEGF receptor gene.

          The invention also features a method of enhancing angiogenesis in a patient involving administering to the patient a non-TNF- $\alpha$  compound which inhibits the TNF- $\alpha$  pathway of downregulating VEGF receptor (e.g., KDR/flk-1  
20 or flt1) gene in an endothelial cell.

          Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, from the drawings, and from the claims.

25                           Detailed Description

          The drawings are first described.

Drawings

          Fig. 1A is a diagram of the human KDR/flk-1 promoter. Restriction enzyme sites are indicated above  
30 the nucleotide sequence, and nucleotide sequences -780 to +487 (SEQ ID NO:7) are numbered on the left of the nucleotide sequence. The transcription start site is indicated by a curved arrow. Potential cis-acting elements are underlined. The PstI sites which were used

- 8 -

to generate the riboprobe are double underlined, and the sequence corresponding to the oligonucleotide which was used for primer extension is underlined with an arrow.

Fig. 1B is a diagram of the murine KDR/flk-1 promoter. Restriction enzyme sites are indicated above the nucleotide sequence. Nucleotide sequences -295 to +205 (SEQ ID NO:8) are numbered and potential cis-acting elements are indicated as in Fig. 1A. An asterisk indicates the 5' end of the cDNA.

Fig. 2A is a photograph of an electrophoretic gel showing the results of a primer extension analysis of the KDR/flk-1 transcription start site. The oligonucleotide underlined with an arrow in Fig. 1A was hybridized to 20  $\mu$ g of total RNA from human umbilical vein endothelial cells (HUVEC) and HeLa cells or 3  $\mu$ g of polyA<sup>+</sup> HUVEC RNA and yeast tRNA. Extension products were analyzed on an 8% polyacrylamide gel (lanes 1-4: Yeast tRNA; HeLa total RNA; HUVEC total RNA; and HUVEC polyA<sup>+</sup> RNA). A Sanger sequencing reaction primed on a plasmid DNA template (with the same oligonucleotide primer) was run next to the primer extension analyses (lanes 5-8: G; A; T; C).

Fig. 2B is a diagram showing the strategy for mapping the transcription start site of the KDR/flk-1 gene by ribonuclease protection.

Fig. 2C is a photograph of an electrophoretic gel showing a ribonuclease protection analysis of the KDR/flk-1 transcription start site. Total RNA from HUVEC and HeLa cells or polyA<sup>+</sup> HUVEC RNA and yeast tRNA were incubated with a 559-bp <sup>32</sup>P-labeled riboprobe spanning the immediate 5' region of the human KDR/flk-1 gene. The annealing products were digested with RNase. Protected fragments were analyzed on a 4% polyacrylamide gel. The size markers (bp) were prepared by radiolabeling  $\Phi$ X174 RF DNA digested with HaeIII.

Fig. 3A is a diagram showing the location of 5' deletion sites in the

- 9 -

KDR/flk-1 promoter. Location of deletion sites is shown in relation to consensus sequences for known nuclear proteins.

Fig. 3B is a bar graph showing the results of a functional analysis of the human KDR/flk-1 promoter by transfection of luciferase reporter constructs containing serial 5' deletions into bovine aortic endothelial cells (BAEC). All constructs were cotransfected with pSV $\beta$ gal to correct for transfection efficiency, and luciferase activity was expressed as a percentage of pGL2 Control (mean  $\pm$  SEM). Fig. 4A is a diagram showing the location of 3' deletion sites in the KDR/flk-1 promoter. Location of deletion sites is shown in relation to consensus sequences for known nuclear proteins.

Fig. 4B is a bar graph showing the results of a functional analysis of 3' deletions on KDR/flk-1 promoter activity in BAEC. Luciferase activity is represented as a percentage of pGL2 control.

Fig. 5 is a bar graph showing the effect of a GATA site mutation on KDR/flk-1 promoter activity. Mutation of the GATA site at position +107 does not decrease the ability of the KDR/flk-1 promoter to direct transcription. When transfected into BAEC, the plasmid pGL2-225+268 directed luciferase expression comparable to that directed by pGL2 Control, which contains the SV40 promoter and enhancer. When three bp of the GATA motif at +107 were mutated to create pGL2 GATA-MUT, there was no significant difference in promoter activity.

Fig. 6A is a photograph of a Northern blot analysis showing that KDR/flk-1 RNA expression is restricted to endothelial cells in culture. RNA was extracted from cells in culture and analyzed by Northern blotting using a human KDR/flk-1 cDNA probe. The following cell types were tested: HUVEC (human umbilical vein endothelial cells), HASMC (human aortic smooth

- 10 -

muscle cells), HISMC (human intestinal smooth muscle cells), fibroblasts (human cultured fibroblasts), RD (human embryonal rhabdomyosarcoma cells) HeLa (human epidermoid carcinoma cells), HepG2 (human hepatoma cells), MCF7 (human breast adenocarcinoma cells), and U937 (human histiocytic lymphoma cells).

Fig. 6B is a photograph of the same agarose gel shown in Fig. 6A which was stained with ethidium bromide (to visualize ribosomal RNA) to show the amount of RNA loaded in each lane.

Fig. 7 is a bar graph showing the results of a luciferase assay. High-level activity of the KDR/flk-1 promoter was found to be specific to endothelial cells. The luciferase reporter construct pGL2-4kb+296 was transfected into cells in culture, and transfection efficiency was assessed by monitoring cotransfection with pSV $\beta$ gal. Results are corrected for transfection efficiency and expressed as a percentage of pGL2 Control activity for each cell type. The following cell types were tested: BAEC, bovine aortic endothelial cells; JEG-3, human choriocarcinoma cells; Saos-2, human osteosarcoma cells; A7r5, rat fetal smooth muscle cells; 3T3, mouse fibroblasts; and HeLa, human epidermoid carcinoma cells.

Fig. 8 is a bar graph showing the effect of TNF- $\alpha$  on VEGF-induced proliferation of HUVEC, as measured by uptake of methyl-[ $^3$ H]thymidine.

Fig. 9A is a photograph of a Northern blot analysis of a time course of TNF- $\alpha$ -induced downregulation of KDR/flk-1 mRNA expression in HUVEC. From left to right, the time points are: 0, 1, 2, 3, 6, 12, and 24 hours, as indicated in Fig. 9B.

Fig. 9B is a bar graph of the results of the time course of TNF- $\alpha$ -induced downregulation of KDR/flk-1 mRNA expression shown in the photograph of Fig. 9A.

- 11 -

Fig. 10A is a photograph of a Northern blot analysis of a dose-response experiment of TNF- $\alpha$ -induced downregulation of KDR/flk-1 mRNA expression in HUVEC. From left to right, 1. 0.1, 1, 10, 50, and 100 ng/ml TNF- $\alpha$  were used, as indicated in Fig. 10B.

Fig. 10B is a bar graph of the results of the dose-response experiment of TNF- $\alpha$ -induced downregulation of KDR/flk-1 mRNA expression in HUVEC shown in the photograph of Fig. 10A.

Fig. 11 is a graph showing the effect of Actinomycin D (ACD) on the levels of KDR/flk-1 RNA in HUVEC.

Fig. 12. is a photograph of immunoprecipitation analysis of KDR/flk-1 protein in HUVEC treated with TNF- $\alpha$  for 0, 12, and 24 hours.

#### Isolation and Characterization of KDR/flk-1 Genomic Clones

##### **Screening of Human and Mouse Genomic Libraries**

A 567-bp human KDR/flk-1 cDNA fragment was generated from HUVEC total RNA by reverse-transcriptase polymerase chain reaction (RT-PCR). This fragment was radiolabeled with [ $\alpha$ - $^{32}$ P]dCTP and used to screen a phage library of human placenta genomic DNA in the vector  $\lambda$ FixII (Stratagene, La Jolla, CA). Likewise, a 451-bp mouse KDR/flk-1 cDNA was generated by RT-PCR from mouse lung total RNA and used to screen a phage library of mouse placenta genomic DNA in the vector  $\lambda$ DashII (Stratagene). Hybridizing clones were isolated and purified from each library, and phage DNA was prepared according to standard procedures.

##### **Cell Culture and mRNA Isolation**

BAEC were isolated and cultured in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (HyClone, Logan,

- 12 -

UT), 600 µg of glutamine/ml, 100 units of penicillin/ml, and 100 µg of streptomycin/ml. Cells were passaged every 3-5 days and cells from passages 4-8 were used for transfection experiments. Saos-2 human osteosarcoma cells (ATCC HTB-85), HeLa human epidermoid carcinoma cells (ATCC CRL-7923), HepG2 human hepatoma cells (ATCC HB-8065), human fibroblasts (ATCC CRL-1634), U937 human histiocytic lymphoma cells (ATCC CRL-7939), RD human embryonal rhabdomyosarcoma cells (ATCC CCL-136), MCF7 human breast adenocarcinoma cells (ATCC HTB-22), JEG-3 human choriocarcinoma cells (ATCC HTB-36), A7r5 fetal rat aortic smooth muscle cells (ATCC CRL-1444), and NIH 3T3 mouse fibroblasts (ATCC CRL-1658) were obtained from the American Type Culture Collection. Primary-culture HUVEC were obtained from Clonetics Corp. (San Diego, CA) and were grown in EGM medium containing 2% fetal calf serum (Clonetics). Primary-culture human aortic and intestinal smooth muscle cells were also obtained from Clonetics Corp. All cells were cultured in conditions identical to those for BAEC, with the exception that medium used for smooth muscle cells was supplemented with 25 mM HEPES (Sigma, St. Louis, MO) and that HUVEC were cultured in EGM medium containing 2% fetal calf serum. Primary-culture cells were passaged every 4-6 days, and cells from passages 3-5 were analyzed. Total RNA from cells in culture was prepared by guanidinium isothiocyanate extraction and centrifugation through cesium chloride.

#### DNA Sequencing

Restriction fragments derived from the human and mouse KDR/flk-1 genomic phage clones were subcloned using standard techniques into pSP72 (Promega, Madison, WI) or pBluescript II SK (Stratagene) and sequenced from ~~alkaline-denatured double-stranded plasmid templates by~~ the dideoxy chain termination method with SEQUENASE® 2.0

- 13 -

DNA polymerase (United States Biochemical, Cleveland, OH). DNA was sequenced from both directions at least twice, and both dGTP and dTTP sequencing protocols were used to resolve compression artifacts in the highly

- 5 GC-rich 5' flanking region of the human and mouse KDR/flk-1 genes. Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI).

#### Primer Extension Analysis

- 10 Primer extension analysis was performed according to known methods, e.g., the method of Fen et al., 1993, Biochemistry 32:7932-7938. A synthetic oligonucleotide primer (5' CTGTCTAGAGAAGGAGGCGCGGAGGTGGA ACT 3'; SEQ ID NO:9) complementary to the 5' end of the human KDR/flk-1
- 15 cDNA (Fig. 1A) was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and hybridized to 20  $\mu$ g of each RNA sample, which was then subjected to reverse transcription. Extension products were analyzed by electrophoresis on an 8% denaturing polyacrylamide gel.

#### 20 Ribonuclease Protection Assay

- A 559-bp PstI-PstI fragment of the human KDR/flk-1 gene (Fig. 2B) was cloned in pSP72 as the template for *in vitro* transcription of an  $\alpha$ -<sup>32</sup>P-labeled antisense RNA with T7 RNA polymerase (Boehringer Mannheim, Indianapolis,
- 25 IN). Gel-purified riboprobe ( $5 \times 10^5$  cpm) was hybridized with 20  $\mu$ g of total RNA or 3  $\mu$ g of polyA RNA plus 17  $\mu$ g of yeast tRNA at 55°C for 16 hours in an annealing buffer containing 20 mM Tris-HCl, pH 7.40, 400 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate in 75% formamide.
- 30 After the RNA had been annealed, the unhybridized RNA was digested for 45 minutes at room temperature with 200 U RNase T1 (Boehringer Mannheim) and 0.3 U RNase A (Boehringer Mannheim) in a buffer containing 10 mM Tris-HCl, pH 7.50, 300 mM NaCl, 5 mM EDTA. The digestion
- 35 products were then treated with proteinase K, extracted

- 14 -

with phenol:chloroform, and analyzed by electrophoresis on a 4% denaturing polyacrylamide gel.

#### Northern Analysis

Total RNA (10  $\mu$ g) from cells in culture was fractionated on a 1.3% formaldehyde-agarose gel and transferred to a nitrocellulose filter. The human KDR/flk-1 cDNA probe was labeled with  $^{32}$ P by random priming, the labeled probe was then used to hybridize the filter. The filter was then autoradiographed for 16 hours on Kodak XAR film at  $-80^{\circ}\text{C}$ .

#### Plasmids

Plasmids pGL2 Basic and pGL2 Control contained the firefly luciferase gene (Promega). pGL2 Basic had no promoter, whereas pGL2 Control was driven by the SV40 promoter and enhancer. The plasmid pSV $\beta$ GAL (Promega) contained the  $\beta$ -galactosidase gene driven by the SV40 promoter and enhancer.

Reporter constructs containing fragments of the human KDR/flk-1 5' flanking region were inserted into pGL2 Basic and named according to the length of the fragment (from the transcription start site) in the 5' and 3' directions. For example, plasmid pGL2-4kb+296 contained a human KDR/flk-1 promoter fragment extending from approximately -4 kb 5' of the transcription start site to position +296 inserted into pGL2 Basic. Plasmids pGL2-4kb+296 and pGL2-900+296 were created by restriction digestion of purified phage DNA by using 5' BamHI and PvuII sites, respectively, and the 3' XhoI site at position +296. Plasmids pGL2-716+268, pGL2-570+268, pGL2-323+268, pGL2-225+268, pGL2-164+268, pGL2-37+268, pGL2-225+127, pGL2-225+105, pGL2-225+56, and pGL2-225+5 were created from promoter fragments generated by PCR of human KDR/flk-1 phage DNA. Plasmids pGL2-116+268, pGL2-95+268, pGL2-77+268, pGL2-60+268, and pGL2-12+268 were created by digesting the promoter fragment contained in



- 15 -

plasmid pGL2-164+268 from the 5' end with exonuclease III (Pharmacia Biotech, Piscataway, NJ). Plasmid pGL2 GATA-MUT was identical to pGL2-225+268 except that bp +108 to +110 were mutated in the plasmid pGL2 GATA-MUT.

- 5 All constructs were sequenced from the 5' and 3' ends to confirm orientation and sequence.

#### Mutagenesis

- Site-directed mutagenesis of the atypical GATA sequence located in the first exon of the human KDR/flk-1 5' flanking region was performed by PCR using to the method of Higushi et al., 1988, Nucleic Acids Res. 16:7351-7367. A DNA fragment containing human KDR/flk-1 bp -225 to +268 was used as a template. The sequence TGGATATC was mutated to TGGTCGTC by using one set of 15 mismatched primers, 5' TCTGGCAGCCTGGTCGTCCTCTCCTA 3' (SEQ ID NO:10) and 5'TAGGAGAGGACGACCAGGCTGCCAGA 3' (SEQ ID NO:11), and one set of primers flanking both ends of the template, 5' TGCCTCGAGTTGTTGCTCTGGGATGTT 3' (SEQ ID NO:12) and 5' TGTAAGCTTGGGAGCCGTTCTTTCTC 3' (SEQ ID 20 NO:13). The sequence of the mutated PCR fragment was confirmed by the dideoxy chain termination method.

#### Transfections

- All cell types were transfected by the calcium phosphate method known in the art with the exception of 25 A7r5 cells, which were transfected with DOTAP (Boehringer Mannheim) as instructed by the manufacturer. In all cases, 20  $\mu$ g of the appropriate reporter construct was transfected along with 2.5  $\mu$ g of pSV $\beta$ gal to correct for variability in transfection efficiency. Cell extracts 30 were prepared 48 hours after transfection by a detergent lysis method (Promega). Luciferase activity was measured in duplicate for all samples with an EG&G Autolumat 953 luminometer (Gaithersburg, MD) and the Promega Luciferase Assay system.  $\beta$ -Galactosidase activity was assayed using

- 16 -

known methods, e.g., Lee et al., 1990, J. Biol. Chem. 265:10446-10450.

The ratio of luciferase activity to  $\beta$ -galactosidase activity in each sample served as a measure of the normalized luciferase activity. The normalized luciferase activity was divided by the activity of pGL2 Control and expressed as relative luciferase activity. Each construct was transfected at least six times, and data for each construct are presented as the mean  $\pm$  SEM. Relative luciferase activity among constructs was compared by a factorial analysis of variance followed by Fisher's least significant difference test. Statistical significance was accepted at  $p < 0.05$ .

#### 15 Isolation and Characterization of Human and Murine KDR/flk-1 Genomic Clones

Initial screening of a human placental phage library with a human KDR/flk-1 cDNA probe yielded a positive clone that was examined by restriction enzyme DNA mapping, subcloning, and sequencing. The 780-bp sequence of the promoter and first exon is shown in Fig. 1A. Likewise, a murine KDR/flk-1 cDNA probe was used to screen a murine placental phage library, and one clone was identified and characterized. The sequence of the mouse KDR/flk-1 promoter is shown in Fig. 1B.

#### 25 Identification of the Transcription Start Site of Human KDR/flk-1

To identify the transcription start site of the human KDR/flk-1 gene, primer extension was performed with a complementary oligonucleotide probe corresponding to bp +212 to +243 (underlined with arrow, Fig. 1A). Primer extension was performed on total RNA from HUVEC and HeLa cells and on polyA RNA from HUVEC. Gene transcription was found to be initiated only in endothelial cells (Fig. 2A). A single transcription start site, corresponding to

- 17 -

a nucleotide located 303 bp 5' of the site of translation initiation, i.e., the methionine initiation codon, was identified. This nucleotide was designated +1. The transcription start site is highlighted in bold in the sequence, CCCTGCACTGA (SEQ ID NO:14) (see Figs. 1A and 2A). The 5'CA3' nucleotide pair at this position is the most common site for transcription initiation.

To confirm the results of the primer extension studies, a ribonuclease protection analysis was performed using an antisense riboprobe generated from a 559-bp genomic PstI-PstI fragment extending 5' from position +146 (Fig. 2B; the PstI sites are double underlined in Fig. 1A). Incubation of this probe with HUVEC polyA RNA and HUVEC total RNA, but not with total RNA from HeLa cells, resulted in protection of a single fragment corresponding in length to the distance between the 3' PstI site and the transcription start site identified by primer extension (Fig. 2C). Despite the absence of a TATA consensus sequence, transcription of the human KDR/flk-1 gene was found to begin from a single site located 303 bp 5' of the translation initiation codon (Fig. 1A, curved arrow).

#### Identification of Cis-Acting Sequences

The 5' flanking sequence of the human KDR/flk-1 gene contains regions rich in G and C residues and lacks TATA and CCAAT boxes near the transcription start site (Fig. 1A). Comparison of this 5' flanking sequence with sequences in the Transcription Factors Database revealed a series of five Sp1 sites located between human KDR/flk-1 nucleotides -124 and -39. There are two AP-2 consensus sites at positions -95 and -68 and two inverted NFkB binding elements at -130 and -83 interspersed among the Sp1 sites. Two atypical GATA consensus sequences (both GGATAT) are present in the KDR/flk-1 promoter, one at position -759 and the other at position +107 within

- 18 -

the untranslated portion of the first exon. In addition, multiple CANNTG elements are located in the promoter at positions -591, -175, +71, and +184; CANNTG elements can be bound by E-box binding proteins. The sequence  
5 AAACCAAA, which is conserved among genes expressed preferentially in keratinocytes, is present at human KDR/flk-1 position -508.

The human and mouse KDR/flk-1 promoters were compared to identify conserved consensus sequences for  
10 nuclear proteins (Fig. 1B). Elements conserved between the two species include two Sp1 sites located at positions -244 and -124 relative to the 5' end of the reported mouse cDNA sequence, two AP-2 sites at positions -168 and -148, a noninverted NFkB site at position -153,  
15 and the keratinocyte element AAACCAAA at position -195. An atypical GATA element (GGATAA) is located in the untranslated portion of the first exon of the mouse promoter at position +18; an atypical GATA element (GGATAT) is located similarly in the human promoter.  
20 Also, a CANNTG sequence is present 12 bp 5' of the G- and C-rich sequences of the promoter at mouse KDR/flk-1 position -257, a location analogous to that of the CANNTG element at position -175 of the human promoter. Conservation of these elements across species suggests  
25 that these regulatory elements have functional significance.

#### Deletion Analysis of the Human KDR/flk-1 Promoter

To identify DNA elements important for basal expression of KDR/flk-1 in endothelial cells, a series of  
30 luciferase reporter plasmids containing serial 5' deletions through the promoter region was constructed (Figs. 3A and 3B). These plasmid constructs in pGL2 Basic were cotransfected into BAEC with pSVβgal (to correct for differences in transfection efficiency) and  
35 the luciferase activity was normalized to that of the

- 19 -

pGL2 Control vector driven by the SV40 promoter/enhancer. The activity of the longest human KDR/flk-1 genomic fragment, spanning bp -4kb to +296, was similar to that of the powerful SV40 promoter/enhancer and consistent with the high level of KDR/flk-1 mRNA expression in endothelial cells. Similar levels of activity were produced in constructs containing as much as 15.5 kb of 5' flanking sequence. Serial 5' deletions from bp -4kb to -225 caused no significant change in promoter activity, implying that elements in this region are not important for basal activity of the KDR/flk-1 promoter. Deletion of sequences between bp -225 and -164 significantly reduced KDR/flk-1 promoter activity to 63% of the activity of the full promoter fragment ( $p < 0.05$ ). These data suggest the presence of positive regulatory elements in this region. Deletion of bp from -95 to -77, a sequence that contains one AP-2 site and one NF $\kappa$ B site, resulted in a further significant decrease in activity to 20% that of pGL2-4kb+296 ( $p < 0.05$ ). Further deletion of bp from -77 to -60, an area containing an overlapping AP-2/Sp1 site, significantly reduced KDR/flk-1 promoter activity to less than 5% that of pGL2-4kb+296 ( $p < 0.05$ ). Thus, 5' deletion analysis revealed that many positive regulatory elements in the KDR/flk-1 promoter are necessary for high-level expression of the gene.

The deletion analyses described above indicate that three sequences within the 5' flanking region of the KDR/flk-1 gene contain elements important for expression in endothelial cells. Potential binding sites for Sp1, AP-2, NF $\kappa$ B, and E-box proteins located within these three positive regulatory elements in the human KDR/flk-1 gene are also present in the mouse 5' flanking sequence, thus suggesting that they are functional binding domains.

AP-2 is a developmentally regulated trans-acting factor (Mitchell et al., 1991, Genes & Dev. 5:105-119) without a

- 20 -

demonstrated role in endothelial cell gene regulation. NFkB is thought to trans-activate the inducible expression of vascular cell adhesion molecule-1 and tissue factor in endothelial cells (Iademarco, 1992, J. Biol. Chem. 267:16323-16329; Moll et al., 1995, J. Biol. Chem. 270:3849-3857) and is known to be a mediator of tissue-specific gene regulation (Lenardo et al., 1989, Cell 58:227-229). Nuclear proteins that bind the E-box motif include the basic helix-loop-helix family of trans-acting factors. E-box binding proteins have not been clearly associated with endothelial cell gene expression, although members of this family are critical for proper maturation of many cell types, including skeletal muscle and B lymphocytes (Buskin et al., 1989, Mol. Cell. Bio. 9:2627-2640; Murre et al., 1989, Cell 58:537-544).

To determine whether sequences in the first exon of human KDR/flk-1 are important for basal expression, a series of 3' deletion constructs from the vector pGL2-225+268, which is the smallest construct that possessed full promoter activity, was made (Figs. 4A and 4B). Deletion of a fragment spanning bp +105 to +127 (SEQ ID NO:4) caused a five-fold reduction in promoter activity ( $p < 0.05$ ), indicating the presence of a positive regulatory element in this region.

The functional importance of the atypical GATA site located between bp +105 and +127 of human KDR/flk-1 was also examined. Three bp of the GATA motif in the fragment -225 to +268 were mutated to GTCG by PCR to create the mutant, pGL2 GATA-MUT. Mutation of these bp in the GATA motif eliminates GATA-2 binding activity in the endothelin-1 gene promoter. In contrast, there was no significant decrease in promoter activity in BAEC with the pGL2 GATA-MUT construct containing the mutated

- 21 -

atypical GATA sequence compared to the native pGL2-225+268 promoter construct, ( $p > 0.05$ ; Fig. 5).

Four zinc finger-containing transcription factors in the GATA protein family bind to the consensus sequence (A/T)GATA(A/G) and regulate cell type-specific gene expression in many cell lineages (Orkin, 1992, Blood 80:575-581); among these, GATA-2 has been most closely linked to endothelial cell gene expression. GATA-2 functions as an enhancer of endothelin-1 gene expression and acts to restrict expression of von Willebrand factor to endothelial cells. Human KDR/flk-1 5' flanking region was found to have two potential GATA-binding sequences, at positions -759 and +107. Loss of the element located at position -759 had no effect on expression of KDR/flk-1 in endothelial cells. The potential GATA element at position +107 is located in a region of the first exon which has now been identified as a powerful positive regulatory element (SEQ ID NO:4). Although this GATA sequence (GGATAT) differs from the GATA-binding sequences of endothelin-1 and von Willebrand factor and from the consensus GATA sequence (A/T)GATA(A/G), the data suggests that it is the functional motif in the region between +105 and +127 because the functional GATA site in the von Willebrand factor gene is located similarly in the first exon, and because a similar GATA element is found in the first exon of the mouse KDR/flk-1 gene. Mutation of three bp in this element (GATA to GTCG), which had been observed to prevent trans-activation of the GATA cis-acting element in the endothelin-1 promoter, was found to have no significant effect on KDR/flk-1 promoter activity (Fig. 5). Thus, the deletion analyses and mutagenesis studies do not support a functional role for the two GATA sequences in the human promoter in its high-level activity in endothelial cells. These observations suggest that transcription factors other

- 22 -

than GATA proteins are necessary for expression of the human KDR/flk-1 gene.

**High-Level Expression Induced by the KDR/flk-1 Promoter Is Specific to Endothelial Cells**

- 5           Although KDR/flk-1 expression is restricted to endothelial cells *in vivo*, it does not necessarily follow that its expression would be limited to endothelial cells in culture. To determine whether a tissue culture system is suitable for studying cell-type specific regulation of
- 10 the KDR/flk-1 gene, Northern analysis of RNA extracted from various cells in culture was performed. KDR/flk-1 message was detected in HUVEC but not in primary-culture cells (human aortic and intestinal smooth muscle cells and fibroblasts) or human cell lines (RD, HeLa, HepG2,
- 15 MCF7, and U937) (see Figs. 6A and 6B). Similarly, KDR/flk-1 message was not detected by RT-PCR in HeLa, A7r5, or 3T3 cells. Thus, expression of KDR/flk-1 message in tissue culture appears to be restricted to endothelial cells, as it is *in vivo*.
- 20           To determine whether 5' flanking sequences of the KDR/flk-1 gene can confer endothelial cell-specific expression in cultured cells, pGL2-4kb+296, which contains over 4 kb of the human KDR/flk-1 5' flanking sequence and includes most of the untranslated portion of
- 25 the first exon, was transfected into a variety of cell types in culture (Fig. 7). Reporter gene expression driven by the pGL2-4kb+296 promoter fragment was similar to that driven by the potent SV40 promoter/enhancer. In JEG-3, Saos-2, A7r5, 3T3, and HeLa cells, however,
- 30 expression driven by the pGL2-4kb+296 promoter was markedly lower, demonstrating that induction of high-level expression by these promoter sequences is specific to endothelial cells. A similar expression pattern was observed using a reporter plasmid containing
- 35 15.5 kb of KDR/flk-1 5' flanking sequence.
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- 23 -

These data indicate that the activity of the KDR/flk-1 promoter in endothelial cells is similar to that of the potent SV40 promoter/enhancer and that this high-level activity is specific to endothelial cells; activity in other cell types is markedly diminished. Low, but detectable, promoter activity was observed in transient transfection assays of cell types that do not express the KDR/flk-1 gene *in vivo*; it is possible that other silencer elements outside of the 15.5 kb 5' flanking region are necessary to block promoter activity completely in non-endothelial cells. Alternatively, the context of the promoter in relation to normal chromatin structure may be essential for precise regulation of the gene. The results described above suggest that tissue-specific regulation of KDR/flk-1 involves a complex interaction between known, widely distributed nuclear factors and other, undefined elements.

#### TNF- $\alpha$ Downregulates KDR/flk-1 and flt1 Expression

##### Cell Culture and mRNA Isolation

Primary-culture HUVEC and HAEC were obtained from Clonetics Corp. (San Diego, CA) and were grown in M199 medium supplemented with 20% fetal calf serum (Hyclone, Logan, UT), 30 mg endothelial cell growth substance (ECGS, Collaborative Biomedical, Bedford, MA), 25 mg heparin, 600  $\mu$ g of glutamine/ml, 100 units of penicillin/ml, and 100  $\mu$ g of streptomycin/ml, in gelatin-coated tissue culture plates. Bovine aortic endothelial cells (BAEC) were isolated and cultured in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum. Primary-culture cells were passaged every 4-6 days and experiments were performed on cells three to six passages from primary culture. After the cells had grown to confluence, they were placed in serum-deprived medium (M199 medium

- 24 -

supplemented with 5% fetal calf serum without ECGS). Recombinant human TNF- $\alpha$  (Genzyme, Cambridge, MA) was aliquoted and stored at -80°C until use. Total RNA from cells in culture was prepared by guanidinium

5 isothiocyanate extraction and centrifugation through cesium chloride (Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

#### 10 Northern Analysis

RNA blots were hybridized as described (Li et al., 1995, J. Biol. Chem. 270:308-312). Total RNA (10  $\mu$ g) from cells in culture was fractionated on a 1.3% formaldehyde-agarose gel and transferred to  
15 nitrocellulose filters. cDNA probes were labeled with  $^{32}$ P by random priming and used to hybridize to the filters. Filters were then washed and subject to autoradiography for 4-8 hours on Kodak XAR film at -80°C. Filters were stripped of radioactive probe in a 50% formamide solution  
20 at 80°C and rehybridized with an end-labeled 18S ribosomal RNA oligonucleotide to correct for loading. Filters were scanned and radioactivity was measured on a PhosphorImager running the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To correct for differences in  
25 RNA loading, the signal intensity for each RNA sample hybridized to the cDNA probes was divided by that for each sample hybridized to the 18S ribosomal RNA probe.

#### Plasmids

A 567-bp human KDR/flk-1 cDNA fragment was  
30 generated from human umbilical vein endothelial cell (HUVEC) total RNA by the reverse-transcriptase polymerase chain reaction (RT-PCR) (Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New  
35 York), as previously described (Patterson et al., 1995,

- 25 -

J. Biol. Chem. 270:2311-23118). The human flt1 cDNA clone was generously provided by Dr. Timothy Quinn (University of California, San Francisco).

#### Nuclear Run-on Analysis

5 Confluent HUVEC were treated with either vehicle (control) or TNF- $\alpha$  (1 ng/ml) for 18 hours. The cells were subsequently lysed, and the nuclei were isolated, as described in Perrella et al. (Perrella et al., 1994, J. Biol. Chem. 269:14595-14600). Nuclear suspension (200  
10  $\mu$ l) was incubated with 0.5 mM each of CTP, ATP, and GTP, and with 20  $\mu$ Ci of  $^{32}$ P-labeled UTP (3000 Ci/mmol, DuPont/NEN, Boston, MA). The samples were extracted with phenol/chloroform, precipitated, and resuspended at equal counts/minute/ml in hybridization buffer ( $15 \times 10^6$   
15 counts/minute/ml). Denatured probes (1  $\mu$ g) dot-blotted onto nitrocellulose filters were hybridized with the samples at 40°C for 4 days in the presence of formamide. cDNAs for the KDR/flk-1 and  $\beta$ -actin genes were used as probes. The filters were scanned and radioactivity was  
20 measured on a PhosphorImager running ImageQuant software. The amount of sample hybridizing to the KDR/flk-1 probe was divided by that hybridizing to the  $\beta$ -actin probe, and the corrected density was reported as the percentage change from the control.

#### 25 Immunoprecipitation

HUVEC in confluent monolayers were serum-deprived for 24 hours and treated with TNF- $\alpha$  (1 ng/ml) or vehicle for the indicated times. The cells were incubated with  $^{35}$ S-methionine (100  $\mu$ Ci/ml, DuPont/NEN) for two hours and  
30 lysed in RIPA buffer at 4°C for ten minutes. After sedimentation of the insoluble fraction, the protein extract was pre-cleaned with Protein A sepharose (0.1  $\mu$ g/ $\mu$ l, Pharmacia Biotech, Piscataway, NJ) for one hour at 4°C followed by centrifugation and collection of  
35 the supernatant. Protein concentrations in the whole

- 26 -

cell lysates were determined by a modified Lowry procedure (DC protein assay; Bio-Rad, Melville, NY) and were confirmed by SDS-polyacrylamide gel fractionation of samples followed by Coomassie Blue staining. Protein samples (500  $\mu$ g) were diluted to 1  $\mu$ g/ $\mu$ l with immunoprecipitation buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1 NP40, 0.5% sodium deoxycholate, 2 mM EDTA, 0.5 mM DTT, 0.02% sodium azide) plus 4 mg/ml BSA, and rocked gently at 4°C for one hour. Specific antibody was added to a concentration of 50  $\mu$ g/ $\mu$ l and the sample was rocked at 4°C for 1.5 hours. Protein A sepharose (10  $\mu$ g) was added and rocking was continued for 1.5 hours. The antigen-antibody-Protein A sepharose conjugates were removed by centrifugation and washed four times with immunoprecipitation buffer. The conjugates were denatured at 100°C for 5 minutes in Laemmli buffer and size fractionated on a 7% SDS-polyacrylamide gel, which was then vacuum-dried and subject to autoradiography. Autoradiograms were scanned with a Howtek Scanmaster 3+ (Hudson, NH) using Adobe Photoshop 3.0, and Scion Image 1.55 was used to quantitate the immunoprecipitated protein.

#### [<sup>3</sup>H]Thymidine Incorporation

HUVEC grown to near confluence in gelatin-coated 24-well tissue culture plates were serum-deprived and pretreated with vehicle or TNF- $\alpha$  (1 ng/ml) for 12 hours before addition of recombinant human VEGF (10 ng/ml, Collaborative) or vehicle. Cells were treated with VEGF for 24 hours and were labeled with methyl-[<sup>3</sup>H]thymidine (DuPont/NEN) at 1  $\mu$ Ci/ml during the last three hours of VEGF treatment. After labeling, the cells were washed with phosphate-buffered saline, fixed in cold 10% trichloroacetic acid, and washed with 95% ethanol. Incorporated [<sup>3</sup>H]thymidine was extracted in 0.2 M NaOH and measured in a liquid scintillation counter. Values

- 27 -

were expressed as the mean  $\pm$  SEM from 6 wells from two separate experiments.

#### Statistical analysis

When appropriate, data from image analyses and  
5 [ $^3\text{H}$ ]thymidine incorporation were expressed as the mean  $\pm$  SEM. Statistical analysis of multiple treatment groups was performed using a factorial analysis of variance followed by Fisher's least significant difference test. Statistical significance was accepted at  $p < 0.05$ .

#### 10 Effect of TNF- $\alpha$ on VEGF-induced Endothelial Cell Proliferation

TNF- $\alpha$  has previously been demonstrated to blunt the mitogenic action of acidic and basic fibroblast growth factors on bovine aortic endothelial cells in a  
15 concentration-dependent manner (Frater-Schroder et al., 1987, Proc. Natl. Acad. Sci. USA 84:5277-5281). To determine whether TNF- $\alpha$  also blocks the proliferative effect of VEGF on human endothelial cells, [ $^3\text{H}$ ]thymidine incorporation was measured as a marker for DNA synthesis  
20 after stimulating HUVEC with human recombinant VEGF. In serum-deprived HUVEC, pretreatment with TNF- $\alpha$  alone in concentrations similar to those used by Frater-Schroder et al. (Frater-Schroder et al., 1987, Proc. Natl. Acad. Sci. USA 84:5277-5281) decreased [ $^3\text{H}$ ]thymidine  
25 incorporation only slightly in comparison to HUVEC pretreated with vehicle (Fig. 8). In comparison to control cells, VEGF treatment alone potently enhanced [ $^3\text{H}$ ]thymidine incorporation in HUVEC by 2.3-fold, as has been previously demonstrated (Connolly et al., 1989, J.  
30 Clin. Invest. 84:1470-1478). However, pre-treatment of HUVEC with TNF- $\alpha$  totally abolished the effect of VEGF on DNA synthesis. These results demonstrate that TNF- $\alpha$  blocks the proliferative response of HUVEC to VEGF.

- 28 -

**Downregulation of VEGF Receptor mRNA by TNF- $\alpha$  in HUVEC**

The 5.7 kb KDR/flk-1 mRNA is constitutively expressed in HUVEC (Patterson et al., 1995, J. Biol. Chem. 270:2311-23118). The 7.0 kb flt1 mRNA is also abundantly expressed by HUVEC in culture. Treatment of HUVEC with TNF- $\alpha$  (10 ng/ml) resulted in a decrease in the mRNA for both receptors (KDR/flk-1 and flt1) that was evident by 6 hours, and that reached 28% and 33% of 0-hour values, respectively, for KDR/flk-1 and flt1 after 24 hours of treatment (Figs. 9A and 9B). That this effect was due to TNF- $\alpha$  alone, and not to serum deprivation, was demonstrated by including a control sample which was serum-deprived for 24 hours and treated with vehicle alone; serum deprivation alone actually slightly induced both KDR/flk-1 and flt1 messages. To exclude the possibility that the downregulation of these two receptors was due to a generalized decrease in mRNA production induced by TNF- $\alpha$ , the same blots were hybridized to a human heparin-binding epidermal growth factor-like factor (HB-EGF) probe. Under these conditions, TNF- $\alpha$  induced a biphasic increase in HB-EGF message, consistent with the results of previous experiments (Yoshizumi et al., 1992, J. Biol. Chem. 267(14):9467-9469). To demonstrate that the effect of TNF- $\alpha$  was not specific to endothelial cells of venous origin, identical experiments were performed with human aortic endothelial cells (HAEC). A similar potent decrease in the message for both receptors was noted in HAEC. The message for KDR/flk-1 was also decreased by TNF- $\alpha$  in bovine aortic endothelial cells, demonstrating that the effect of TNF- $\alpha$  is not species-specific.

TNF- $\alpha$  also decreased the messages for KDR/flk-1 and flt1 in a dose-dependent fashion (Figs. 10A and 10B). As little as 1 ng/ml TNF- $\alpha$  inhibited the mRNA for both

- 29 -

receptors to near maximal levels, with KDR/flk-1 being slightly more sensitive than flt1 to the effects of TNF- $\alpha$  in HUVEC. Thus, TNF- $\alpha$  specifically downregulates the mRNA for the VEGF receptors KDR/flk-1 and flt1 in a time and dose-dependent fashion in human endothelial cells. **TNF- $\alpha$  Decreased the Rate of Transcription, But Had No Effect on the Half-Life, of KDR/flk-1**

To determine whether TNF- $\alpha$  affected the steady-state level of KDR/flk-1 mRNA by increasing its rate of degradation, KDR/flk-1 mRNA was measured in the presence of actinomycin D (ACD; 5  $\mu$ g/ml). The KDR/flk-1 mRNA half-life was 1.9 hours in the absence of TNF- $\alpha$  and increased slightly, to 2.6 hours, in the presence of TNF- $\alpha$  (Fig. 11). In similar experiments, the mRNA half-life of flt1 was found not to be decreased by TNF- $\alpha$  in HUVEC. Thus, the TNF- $\alpha$ -induced decrease in the level of KDR/flk-1 and flt1 mRNAs in HUVEC was not due to a decrease in the stability of the mRNA.

Nuclear run-on experiments were performed to determine the rate of KDR/flk-1 gene transcription in the presence or absence of TNF- $\alpha$ , and to compare it with the rate of transcription of the constitutively expressed  $\beta$ -actin gene. TNF- $\alpha$  decreased the rate of KDR/flk-1 gene transcription (measured in PhosphorImager units) to 40% of baseline, but had no effect on the transcription of  $\beta$ -actin. Thus, the TNF- $\alpha$ -induced decrease in KDR/flk-1 mRNA was due to a decrease in the rate of transcription of the gene in HUVEC and not to a change in the stability of the mRNA.

**The Decrease in KDR/flk-1 mRNA by TNF- $\alpha$  is Protein Synthesis-Dependent**

Whether the decrease in KDR/flk-1 mRNA required protein synthesis was examined using the protein synthesis inhibitor anisomycin. Concentrations of anisomycin used (50  $\mu$ M) were five times higher than those

- 30 -

which inhibit protein synthesis in HUVEC by greater than 95%, as measured by [<sup>3</sup>H]leucine uptake. Anisomycin alone had little effect on KDR/flk-1 expression at this dose. However, pretreatment with anisomycin significantly  
5 blunted the effect of TNF- $\alpha$  on KDR/flk-1 expression (29% vs. 65%,  $p < 0.05$ ), indicating that the effect of TNF- $\alpha$  on KDR/flk-1 in HUVEC was at least partly dependent on new protein synthesis. These results did not vary with the protein synthesis inhibitor used, as cycloheximide caused  
10 an identical inhibition of the TNF- $\alpha$  effect. Our studies also show that the effect of TNF- $\alpha$  on flt1 expression is similarly protein synthesis-dependent in HUVEC.

#### **TNF- $\alpha$ Decreased New KDR/flk-1 Protein Synthesis in HUVEC**

Immunoprecipitation of <sup>35</sup>S-labeled HUVEC lysates  
15 was performed to demonstrate the production of immunoreactive KDR/flk-1 protein by HUVEC and to determine whether the decrease in KDR/flk-1 mRNA was accompanied by a decrease in protein synthesis. A rabbit anti-human KDR/flk-1 antibody (Santa Cruz SC-xxx)  
20 immunoprecipitated a single species with a molecular mass of approximately 205 kDa, consistent with the size of full length KDR/flk-1 protein when expressed in, and immunoprecipitated from, NIH 3T3 or COS7 cells (Quinn et al., 1993, Proc. Natl. Acad. Sci. USA 90(16):7533-7537;  
25 Millauer et al., 1994, Nature 367(6463):576-579). An identically sized species was detected by an antibody raised against a different KDR/flk-1 epitope (Santa Cruz SC-xyx), but not by a rabbit antibody raised in a similar fashion to the transcription factor Sp1,  
30 demonstrating the specificity of this interaction. Treatment of HUVEC with TNF- $\alpha$  for 12 hours increased <sup>35</sup>S-labeled KDR-flk-1 protein levels slightly (<40%), but reproducibly, raising the intriguing possibility that TNF- $\alpha$  also regulates KDR/flk-1 at the translational or  
35 post-translational level. After 24 hours of TNF- $\alpha$



- 31 -

treatment, <sup>35</sup>S-labeled KDR/flk-1 protein levels were decreased to 18% of control levels, confirming that the decrease in KDR/flk-1 mRNA induced by TNF- $\alpha$  is accompanied by a similar decrease in KDR/flk-1 protein expression (Fig. 12).

### Use

The DNA of the invention promotes endothelial cell-specific transcription of DNA sequences to which it is operably linked. These promoter sequences are useful to direct or prevent the expression of genes specifically in endothelial cells. The invention provides the basis for novel therapeutic approaches to vascular diseases such as arteriosclerosis, as well as non-vascular diseases such as cancer, e.g., solid tumors, and inflammatory diseases, e.g., rheumatoid arthritis and diabetic retinopathy, as described in Examples 1 and 2 below.

The invention also provides methods for identifying compounds which (1) modulate TNF- $\alpha$  downregulation VEGF receptor (e.g., KDR/flk-1 or flt1) gene expression (Example 3, below), or (2) modulate TNF- $\alpha$  inhibition of VEGF-induced endothelial cell proliferation (Example 4). Compounds found to enhance TNF- $\alpha$  downregulation of expression of a VEGF receptor gene or enhance TNF- $\alpha$  inhibition of VEGF-induced endothelial cell proliferation can be used in methods to inhibit angiogenesis, while compounds found to enhance TNF- $\alpha$  downregulation of KDR/flk-1 or enhance TNF- $\alpha$  inhibition of VEGF-induced endothelial cell proliferation can be used in methods to promote angiogenesis, for example, to promote wound healing (e.g., healing of broken bones, burns, diabetic ulcers, and traumatic or surgical wounds) or to treat peripheral vascular disease, atherosclerosis, cerebral vascular disease, hypoxic tissue damage (e.g.,

- 32 -

hypoxic damage to heart tissue), diabetic pathologies such as chronic skin lesions, or coronary vascular disease. These compounds can also be used to treat patients who have, or have had, transient ischemic attacks, vascular graft surgery, balloon angioplasty, frostbite, gangrene, or poor circulation. As is described in Example 5, identification of the cis-acting sequences in the KDR/flk-1 gene required for downregulation by TNF- $\alpha$  provides the basis for additional therapeutic methods for these conditions.

#### Example 1: Gene Therapy

The invention can be used for gene therapy treatment of vascular diseases. The DNA of the invention can be used alone or as part of a vector to express heterologous genes, e.g., genes which encode proteins other than KDR/flk-1, in cells of the blood vessel wall, i.e., endothelial cells, for gene therapy of vascular diseases such as arteriosclerosis. The DNA or vector containing a sequence encoding a polypeptide of interest is introduced into endothelial cells which in turn produce the polypeptide of interest. For example, sequences encoding t-PA (Pennica et al., 1982, Nature 301:214), p21 cell cycle inhibitor (El-Deiry et al., 1993, Cell 75:817-823), or nitric oxide synthase (Bredt et al., 1990, Nature 347:768-770) may be operably linked to the endothelial cell-specific promoter sequences of the invention and expressed in endothelial cells. For example, thrombolytic agents can be expressed under the control of the endothelial cell-specific promoter sequences for expression by vascular endothelial cells in blood vessels, e.g., vessels occluded by aberrant blood clots. Other heterologous proteins, e.g., proteins which inhibit smooth muscle cell proliferation, e.g., interferon- $\gamma$  and atrial natriuretic polypeptide, may be

- 33 -

specifically expressed in endothelial cells to ensure the delivery of these therapeutic peptides to an arteriosclerotic lesion or an area at risk of developing an arteriosclerotic lesion, e.g., an injured blood vessel.

The endothelial cell-specific promoter sequences of the invention may also be used in gene therapy to promote angiogenesis to treat diseases such as peripheral vascular disease or coronary artery disease (Isner et al., 1995, Circulation 91:2687-2692). For example, the DNA of the invention can be operably linked to sequences encoding cellular growth factors which promote angiogenesis, e.g., VEGF, acidic fibroblast growth factor, or basic fibroblast growth factor.

According to the invention, the DNA of the invention is located sufficiently close to the coding sequence to be transcribed that it functions to direct expression of the polypeptide in an endothelial cell. For example, SEQ ID NO:1, 2, and 3 are preferably located 5' to the transcription start site, and SEQ ID NO:4 is located 3' of the transcription start site. However, these sequences may be in any order relative to the transcription start site provided that endothelial cell-specific promoter activity is preserved.

#### Example 2: Antisense Therapy

The DNA of the invention may also be used in methods of antisense therapy. Antisense therapy may be carried out by administering to an animal, e.g., a human patient, DNA containing the endothelial cell-specific promoter sequences of the invention operably linked to a DNA sequence, i.e., an antisense template, which is transcribed into an antisense RNA. The antisense RNA may be a short (generally at least 10, preferably at least 14 nucleotides, and up to 100 or more nucleotides) nucleotide sequence formulated to be complementary to a

- 34 -

portion of a specific mRNA sequence. The antisense template is preferably located downstream from the promoter sequences of the invention. A poly A tail is typically located at the end of the antisense sequence to  
5 signal the end of the sequence. Standard methods relating to antisense technology have been described (Melani et al., Cancer Res. 51:2897-2901, 1991). Following transcription of the DNA sequence into antisense RNA, the antisense RNA binds to its target mRNA  
10 molecules within a cell, thereby inhibiting translation of the mRNA and down-regulating expression of the protein encoded by the mRNA. For example, an antisense sequence complementary to a portion of or all of the KDR-flk-1 mRNA (Terman et al., 1991, Oncogene 6:1677-1683) would  
15 inhibit the expression of KDR-flk-1, which in turn would inhibit angiogenesis. Such antisense therapy may be used to treat cancer, particularly to inhibit angiogenesis at the site of a solid tumor, as well as other pathogenic conditions which are caused by or exacerbated by  
20 angiogenesis, e.g., inflammatory diseases such as rheumatoid arthritis, and diabetic retinopathy.

The expression of other endothelial cell proteins may also be inhibited in a similar manner. For example, the DNA of the invention can be operably linked to  
25 antisense templates which are transcribed into antisense RNA capable of inhibiting the expression of the following endothelial cell proteins: cell cycle proteins (thereby inhibiting endothelial cell proliferation, and therefore, angiogenesis); coagulation factors such as von Willebrand  
30 factor; and endothelial cell adhesion factors, such as ICAM-1 and VCAM-1 (Bennett et al., 1994, J. Immunol. 152:3530-3540).

For gene therapy or antisense therapy, the  
35 ~~claimed DNA may be introduced into target cells of an~~  
animal, e.g., a patient, using standard vectors and/or

- 35 -

gene delivery systems. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others. Delivery of nucleic acids to a specific site in the body for gene therapy or antisense therapy may also be accomplished using a biolistic delivery system, such as that described by Williams et al., 1991, Proc. Natl. Acad. Sci. USA 88:2726-2729.

Standard methods for transfecting cells with isolated DNA are well known to those skilled in the art of molecular biology. Gene therapy and antisense therapy to prevent or decrease the development of arteriosclerosis or inhibit angiogenesis may be carried out by directly administering the claimed DNA to a patient or by transfecting endothelial cells with the claimed DNA ex vivo and infusing the transfected cells into the patient.

DNA or transfected cells may be administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal, e.g., physiological saline. A therapeutically effective amount is an amount of the DNA of the invention which is capable of producing a medically desirable result in a treated animal. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for intravenous administration of DNA is from approximately  $10^6$  to  $10^{22}$  copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA

- 36 -

may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

Example 3: Identification of Compounds Which Modulate

5 TNF- $\alpha$  Downregulation of VEGF Receptor (KDR/flk-1 or flt1)  
Gene Expression

As is discussed above, TNF- $\alpha$  downregulates expression of KDR/flk-1 and flt1 genes, each of which encodes a VEGF receptor. Thus, potentiating TNF- $\alpha$   
10 downregulation of KDR/flk-1 or flt1 expression can be useful in decreasing endothelial cell growth and, therefore, in inhibiting processes such as angiogenesis. Conversely, inhibiting TNF- $\alpha$  downregulation of KDR/flk-1 or flt1 expression can be useful in increasing  
15 endothelial cell growth in order to promote angiogenesis, as would be desirable in promoting wound healing or in the treatment of peripheral vascular disease.

Modulation of endothelial cell growth can be achieved by administering a compound which blocks or  
20 enhances TNF- $\alpha$ -mediated inhibition of KDR/flk-1 or flt1 expression. Such a compound can be identified by methods ranging from rational drug design to screening of random compounds. The latter method is preferable, as a simple and rapid assay for carrying out the method is available.  
25 Small organic molecules are desirable candidate compounds for this analysis, as frequently these molecules are capable of passing through the plasma membrane so that they can potentially modulate TNF- $\alpha$  regulation of KDR/flk-1 or flt1 gene expression within the cell.

30 The screening of small, membrane-permeable organic molecules for the ability to modulate TNF- $\alpha$  downregulation of KDR/flk-1 or flt1 is carried out as follows. Cells expressing KDR/flk-1 or flt1 (e.g., HUVEC) are cultured in the presence of TNF- $\alpha$  and the  
35 candidate compound. (Cells containing the KDR/flk-1 (or

- 37 -

flt1) promoter operably linked to a reporter gene may also be used in this method, provided that the promoter is active in the cells in the absence of TNF- $\alpha$ .) The level of KDR/flk-1 (or flt1) expression (as measured by, 5 e.g., Northern blot analysis (see above), RNase protection analysis, or other standard methods) in these cells is compared to the level in cells cultured with TNF- $\alpha$ , but without the candidate compound.

An increase in KDR/flk-1 (or flt1) expression 10 indicates identification of a compound which blocks TNF- $\alpha$  downregulation of KDR/flk-1 (or flt1) expression. As is mentioned above, such a compound can be used in the treatment of conditions in which enhancement of endothelial cell growth or angiogenesis is desired, e.g., 15 peripheral vascular disease, as well as for promoting wound healing. One specific condition in which angiogenesis is desired involves interruption of cardiac blood flow. In such situations, TNF- $\alpha$  may hinder the natural angiogenic process which could control damage to 20 cardiac tissue.

A decrease in KDR/flk-1 (or flt1) expression indicates identification of a compound which potentiates TNF- $\alpha$  downregulation of KDR/flk-1 expression. Such a compound can be used to treat conditions in which 25 decreased endothelial cell growth or angiogenesis is desired. For example, the growth of a tumor may be inhibited by treatment with such a compound.

Compounds identified as having the desired effect (i.e., enhancing or inhibiting TNF- $\alpha$  downregulation of 30 KDR/flk-1 or flt1 expression) can be tested further in appropriate models of endothelial cell growth and angiogenesis which are known to those skilled in the art.

The therapeutic compounds identified using the method of the invention may be administered to a patient 35 by any appropriate method for the particular compound,

- 38 -

e.g., orally, intravenously, parenterally, transdermally, transmucosally, or by surgery or implantation (e.g., with the compound being in the form of a solid or semi-solid biologically compatible and resorbable matrix) at or near  
5 the site where the effect of the compound is desired. For example, a salve or transdermal patch that can be directly applied to the skin so that a sufficient quantity of the compound is absorbed to increase vascularization locally may be used. This method would  
10 apply most generally to wounds on the skin. Salves containing the compound can be applied topically to induce new blood vessel formation locally, thereby improving oxygenation of the area and hastening wound healing. Therapeutic doses are determined specifically  
15 for each compound, most administered within the range of 0.001 to 100.0 mg/kg body weight, or within a range that is clinically determined to be appropriate by one skilled in the art.

Example 4: Identification of Compounds Which Modulate  
20 the Effect of TNF- $\alpha$  on VEGF-Induced Epithelial Cell Growth

As is discussed above, TNF- $\alpha$  inhibits VEGF-induced proliferation of endothelial cells (see, e.g., Fig. 8 and the corresponding text). Accordingly, compounds which  
25 modulate the effect of TNF- $\alpha$  on VEGF-induced endothelial cell growth can be used to treat conditions associated with endothelial cell growth, such as angiogenesis. Such compounds can be identified using the methods described above. For example, endothelial cells can be cultured  
30 with VEGF and TNF- $\alpha$  in the presence and absence of the candidate compound in order to determine whether the compound affects endothelial cell growth, which can be measured, e.g., by monitoring uptake of [ $^3$ H]thymidine.  
As is discussed above, compounds found to have the  
35 desired effect (i.e., enhancing or inhibiting TNF- $\alpha$



- 39 -

inhibition of VEGF-induced endothelial cell proliferation) can be tested further in appropriate models of endothelial cell growth and angiogenesis, which are known to those skilled in the art. Compounds

5 identified using this method are administered to patients as is described above in Example 3. This method may also be carried out without the addition of VEGF, in order to identify compounds which modulate the effect of TNF- $\alpha$  on the growth of endothelial cells in the absence of VEGF.

10 Example 5: Identification of the Cis-Acting Element in the KDR/flk-1 Gene Required for TNF- $\alpha$  Downregulation

Identification of the cis-acting element in the KDR/flk-1 gene required for TNF- $\alpha$ -mediated downregulation (the TNF- $\alpha$ -responsive element), as well as the trans-  
15 acting factor which interacts with the TNF- $\alpha$ -responsive element, will form the basis for the development of novel therapeutics for modulating conditions associated with endothelial cell growth, such as angiogenesis, vascular disease, and wound healing.

20 Identification of the cis-acting elements of a gene, as well as the corresponding trans-acting factors, are carried out using standard methods in the art (see, e.g., Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor  
25 Laboratory Press, Cold Spring Harbor, New York; Ausubel et al., eds., Current Protocols in Molecular Biology, Wiley & Sons, New York, 1989). For example, as a starting point, DNase I hypersensitivity experiments can be carried out in order to identify regions in the gene  
30 which potentially bind regulatory factors.

Identification of the precise sequences of the cis-acting element (e.g., the TNF- $\alpha$  responsive element in the KDR/flk-1 gene) can be carried out using standard promoter deletion analysis. A construct including  
35 KDR/flk-1 sequences that confer TNF- $\alpha$  downregulation to a

- 40 -

reporter gene to which the sequences are operably linked, can be progressively deleted, by 5', 3', and/or nested deletions, until the effect of TNF- $\alpha$  on the expression of the reporter gene in transfected cells is reduced.

- 5 Promoter deletion constructs, such as those described above, can be used to begin this analysis. To confirm the identification of the TNF- $\alpha$ -responsive element identified in the deletion studies, point mutations can be introduced into the element, using standard methods,  
10 in the context of the full promoter.

- The KDR/flk-1 TNF- $\alpha$ -responsive element can then be used as a tool for identifying trans-acting factors which bind to it, and thus are likely to be components of the pathway of TNF- $\alpha$  downregulation of KDR/flk-1. To  
15 determine whether a protein binds to the element, standard DNA footprinting and/or native gel-shift analyses can be carried out. In order to identify the trans-acting factor which binds to the TNF- $\alpha$ -responsive element, the element can be used as an affinity reagent  
20 in standard protein purification methods, or as a probe for screening an expression library. Once the trans-acting factor is identified, modulation of its binding to the TNF- $\alpha$ -responsive element in the KDR/flk-1 gene can be pursued, beginning with, for example, screening for  
25 inhibitors of trans-acting factor binding. Enhancement of TNF- $\alpha$  downregulation of KDR/flk-1 expression in a patient, and thus inhibition of angiogenesis, may be achieved by administration of the trans-acting factor, or the gene encoding it (e.g., in a vector for gene  
30 therapy). In addition, if the active form of the trans-acting factor is a dimer, dominant-negative mutants of the trans-acting factor could be made in order to inhibit its activity. Furthermore, upon identification of the TNF- $\alpha$ -responsive element in the KDR/flk-1 gene, and its  
35 corresponding trans-acting factor, further components in

- 41 -

the TNF- $\alpha$  pathway of KDR/flk-1 downregulation can be identified. Modulation of the activities of these components can then be pursued, in order to develop additional drugs and methods for modulating endothelial cell growth and angiogenesis. The methods described in this example can also be carried out with the flt1 gene.

#### Other Embodiments

In addition to antisense therapy for inhibition of angiogenesis, expression of KDR/flk-1 in endothelial cells can also be carried out by inhibiting the binding of transcription factors, e.g., AP-2, SP-1 and NF $\kappa$ B, to the cis-acting binding sites in the promoter sequences of the invention. For example, transcription can be inhibited using dominant negative mutants of transcription factors, e.g., a dominant negative mutant of AP-2 which binds to the AP-1 binding site but fails to activate transcription. Alternatively, compounds which downregulate production of transcription factors, e.g., retinoic acid or dexamethasone which downregulate production of AP-2 and NF $\kappa$ B, can be administered to inhibit angiogenesis by inhibiting expression of KDR/flk-1.

- 42 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: President and Fellows of Harvard College
- (ii) TITLE OF INVENTION: TRANSCRIPTIONAL REGULATION OF GENES ENCODING VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
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  - (B) STREET: 225 Franklin Street
  - (C) CITY: Boston
  - (D) STATE: MA
  - (E) COUNTRY: USA
  - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
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  - (B) FILING DATE: DEC-18-1995
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  - (A) APPLICATION NUMBER: 08/494,282
  - (B) FILING DATE: JUN-23-1995
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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  - (B) REGISTRATION NUMBER: 34,819
  - (C) REFERENCE/DOCKET NUMBER: 05433/021001
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617/542-5070
  - (B) TELEFAX: 617/542-8906
  - (C) TELEX: 200154

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 62 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: both
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: DNA
-

- 43 -

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GG 62

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGGCCGCA CGGGAGAGC 19

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGATATCCTC TCCTACCGGC AC 22

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 493 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both

- 44 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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CCCCGCCTCC GCGCTCTAGA GTTTCGGCTC CAGCTCCCAC CCTGCACTGA GTCCCGGGAC	240
CCCGGGAGAG CGGTCACTGT GTGGTCGCTG CGTTTCCTCT GCCTGCGCCG GGCATCACTT	300
GCGCGCCGCA GAAAGTCCGT CTGGCAGCCT GGATATCCTC TCCTACCGGC ACCCGCAGAC	360
GCCCCTGCAG CCGCCGGTCG GCGCCGGGCG TCCCTAGCCC TGTGCGCTCA ACTGTCCTGC	420
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AGAACCGGCT CCC	493

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 352 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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GAGGGGCGGG GCTGGCCGCA CGGGAGAGCC CCTCCTCCGC CCCGGCCCCG CCCCGCATGG	180
CCCCGCCTCC GCGCTCTAGA GTTTCGGCTC CAGCTCCCAC CCTGCACTGA GTCCCGGGAC	240
CCCGGGAGAG CGGTCACTGT GTGGTCGCTG CGTTTCCTCT GCCTGCGCCG GGCATCACTT	300
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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1267 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- 45 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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CAGTCCAGTT GTGTGGGGAA ATGGGGAGAT GTAAATGGGC TTGGGGAGCT GGAGATCCCC	660
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CCCCGCCCCG CATGGCCCCG CCTCCGCGCT CTAGAGTTTC GGCTCCAGCT CCCACCCTGC	780
ACTGAGTCCC GGGACCCCGG GAGAGCGGTC AGTGTGTGGT CGCTGCGTTT CCTCTGCCTG	840
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CAGGCGCTGG GAGAAAGAAC CGGCTCCCGA GTTCTGGGCA TTTCGCCCGG CTCGAGGTGC	1080
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GACATT	1267

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 500 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

- 46 -

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GTGGGGGGCG TGGCCGGACG CAGGGAGTCC CCACCCCTCC CGGTAATGAC CCCGCCCCCA	180
TTGCTAGTG TGTAGCCGGC GCTCTCTTTC TGCCCTGAGT CCTCAGGACC CCAAGAGAGT	240
AAGCTGTGTT TCCTTAGATT CGGGGACCGC TACCCGGCAG GACTGAAAGC CCAGACTGTG	300
TCCCGCAGCC GGGATAACCT GGCTGACCCG ATTCGCGGGA CACCGCTGCA GCCGCGGCTG	360
GAGCCAGGGC GCCGGTGCCC CGCGCTCTCC CCGGTCTTGC GAAGGAGTCT GTGCCTGAGA	420
AACTGGGCTC TGTGCCCAGG CGCGAGGTGC AGGATGGAGA GCAAGGCGCT GCTAGCTGTC	480
GCTCTGTGGT TCTGCGTGGA	500

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGTCTAGAG AAGGAGGCGC GGAGGTGGAA CT	32
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## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCTGGCAGCC TGGTCGTCCT CTCCTA	26
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## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA



- 47 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAGGAGAGGA CGACCAGGCT GCCAGA

26

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGCCTCGAGT TGTGCTCTG GGATGTT

27

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGTAAGCTTG GGAGCCGGTT CTTTCTC

27

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 11 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCCTGCACTG A

11

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

- 48 -

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Gln Ser Lys Val Leu Leu Ala Val Ala Leu Trp Leu Cys Val Glu  
1 5 10 15

Thr Arg Ala Ala Ser Val  
20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Glu Ser Lys Ala Leu Leu Ala Val Ala Leu Trp Phe Cys Val Lys  
1 5 10 15

Other embodiments are within the following claims.

What is claimed is:

-49-

## CLAIMS:

1. A substantially pure DNA comprising a sequence substantially identical to SEQ ID NO:1, wherein said DNA regulates endothelial cell-specific transcription of a polypeptide-encoding sequence to which  
5 it is operably linked.
2. The DNA of claim 1, wherein said DNA further comprises a sequence substantially identical to SEQ ID NO:2 or a sequence substantially identical to SEQ ID NO:3.
- 10 3. The DNA of claim 1, wherein said DNA further comprises a sequence substantially identical to SEQ ID NO:4.
4. The DNA of claim 2, wherein said DNA further comprises a sequence substantially identical to SEQ ID  
15 NO:4.
5. A substantially pure DNA comprising a sequence substantially identical to SEQ ID NO:6, wherein said DNA regulates endothelial cell-specific transcription of a polypeptide-encoding sequence to which  
20 it is operably linked.
6. A substantially pure DNA comprising a sequence substantially identical to SEQ ID NO:5, wherein said DNA regulates endothelial cell-specific transcription of a polypeptide-encoding sequence to which  
25 it is operably linked.
7. The DNA of claim 1, wherein said DNA is operably linked to said polypeptide-encoding sequence and functions to regulate endothelial cell-specific transcription of said polypeptide-encoding sequence.

- 50 -

8. The DNA of claim 7, wherein said polypeptide-encoding sequence does not encode KDR/flk-1.

9. The DNA of claim 8, wherein said polypeptide is chosen from a group consisting of tissue plasminogen  
5 activator, p21 cell cycle inhibitor, nitric oxide synthase, interferon- $\gamma$ , and atrial natriuretic polypeptide.

10. A vector comprising the DNA of claim 8.

11. A method of directing endothelial cell-  
10 specific expression of a polypeptide, comprising introducing into an endothelial cell the vector of claim 10.

12. An endothelial cell comprising the vector of claim 10.

15 13. A method of inhibiting arteriosclerosis in an animal, comprising contacting an artery of said animal with the vector of claim 10, wherein said polypeptide reduces or prevents the development of arteriosclerosis.

14. The method of claim 13, wherein said  
20 polypeptide reduces proliferation of smooth muscle cells.

15. A substantially pure DNA with a sequence substantially identical to SEQ ID NO:1, wherein said DNA regulates endothelial cell-specific transcription of an antisense template to which it is operably linked.

25 16. The DNA of claim 15, wherein said DNA is operably linked to said antisense template and wherein

- 51 -

said DNA functions to regulate endothelial cell-specific transcription of said antisense template.

17. The DNA of claim 16, wherein said antisense template is complementary to an mRNA encoding an  
5 endothelial cell polypeptide.

18. The DNA of claim 14, wherein said endothelial cell polypeptide is KDR/flk-1.

19. The DNA of claim 17, wherein said endothelial cell polypeptide is chosen from a group consisting of a  
10 cell cycle protein, a coagulation factor, and a cell adhesion factor.

20. A method of treating cancer in an animal, comprising contacting a tumor site in said animal with the DNA of claim 18, wherein said DNA reduces or prevents  
15 angiogenesis at said tumor site.

21. A method of measuring the ability of a candidate compound to modulate TNF- $\alpha$  downregulation of expression of a vascular endothelial growth factor receptor gene, said method comprising the steps of:

20 (a) providing a cell comprising the promoter of said vascular endothelial growth factor receptor gene operably linked to a reporter gene;

(b) culturing said cell in the presence of TNF- $\alpha$  and said candidate compound; and

25 (c) determining the level of expression of said reporter gene as a measure of the ability of said candidate compound to modulate TNF- $\alpha$  downregulation of expression of said vascular endothelial growth factor receptor gene.

- 52 -

22. A method of measuring the ability of a candidate compound to modulate TNF- $\alpha$  inhibition of VEGF-induced endothelial cell proliferation, said method comprising the steps of:

- 5 (a) providing an endothelial cell;
- (b) culturing said cell in the presence of TNF- $\alpha$ , VEGF, and said candidate compound; and
- (c) determining the level of endothelial cell growth as a measure of the ability of said candidate
- 10 compound to modulate TNF- $\alpha$  inhibition of VEGF-induced endothelial cell proliferation.

23. A method of inhibiting angiogenesis in a patient, said method comprising administering to said patient a non-TNF- $\alpha$  compound which activates the TNF- $\alpha$

15 pathway of downregulating expression of a vascular endothelial growth factor receptor gene in an endothelial cell.

24. A method of enhancing angiogenesis in a patient, said method comprising administering to said

20 patient a non-TNF- $\alpha$  compound which inhibits the TNF- $\alpha$  pathway of downregulating expression of a vascular endothelial growth factor receptor gene in an endothelial cell.

25. A method of inhibiting angiogenesis in a

25 patient, said method comprising administering to said patient a polypeptide which inhibits expression of a vascular endothelial cell growth factor receptor gene in an endothelial cell by binding to the TNF- $\alpha$ -responsive element in the promoter of said vascular endothelial cell

30 growth factor receptor gene.

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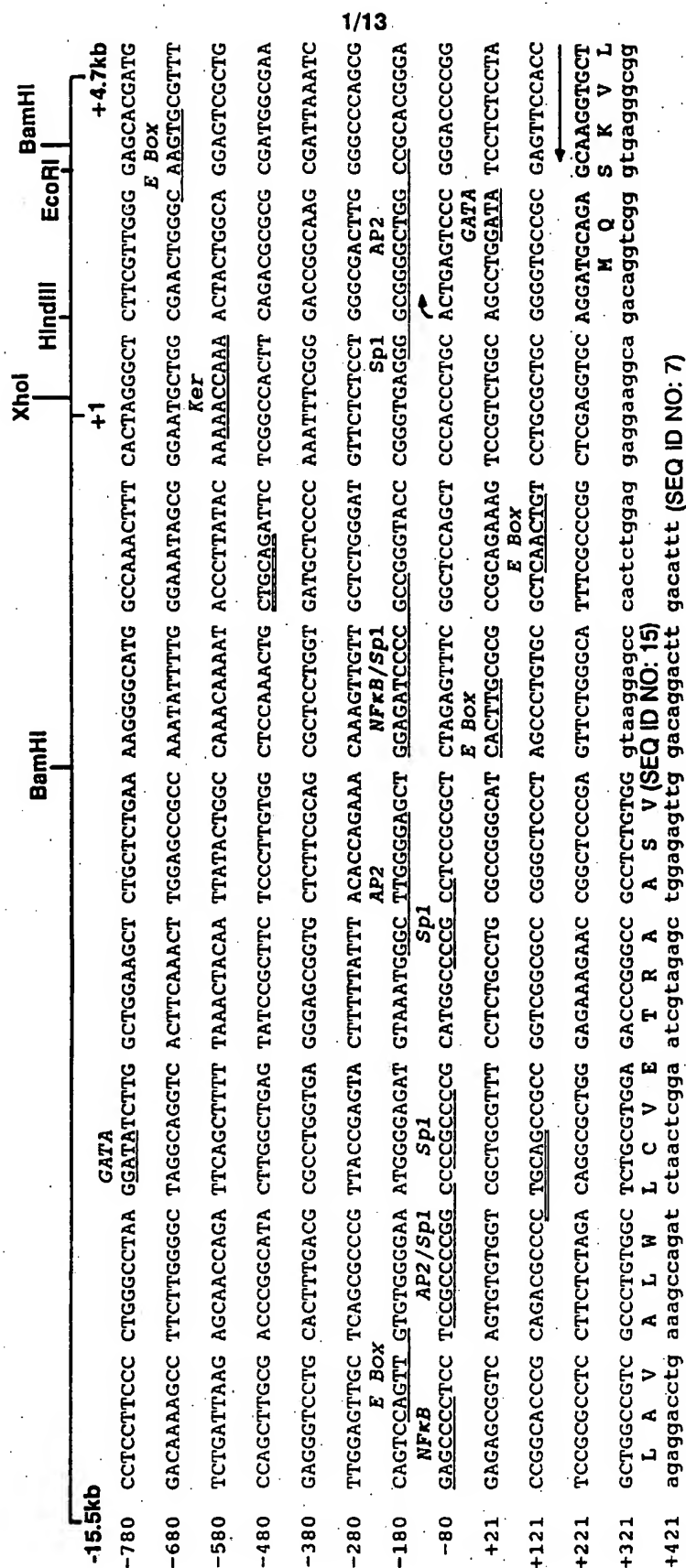


FIG. 1A

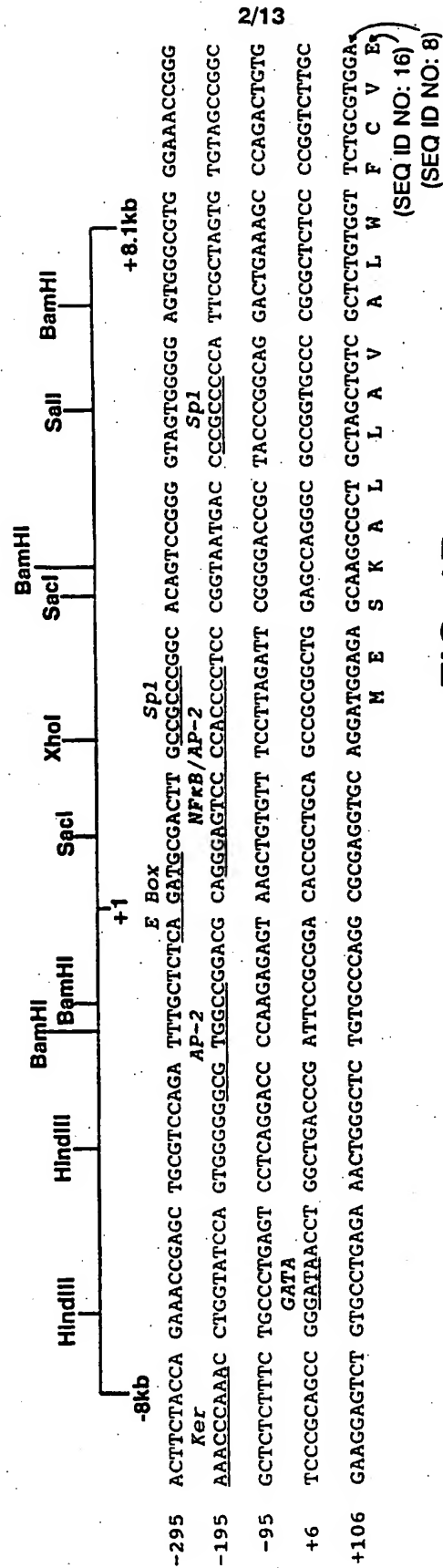


FIG. 1B



3/13

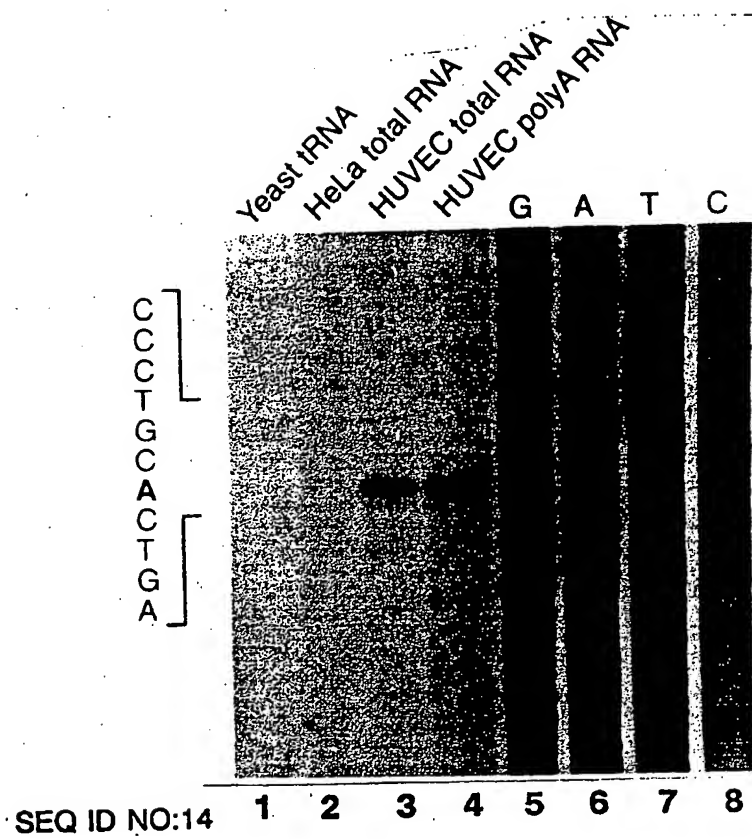
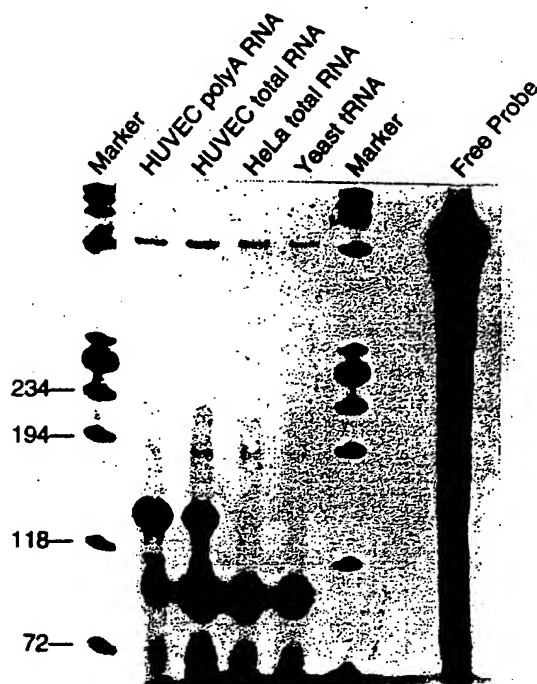
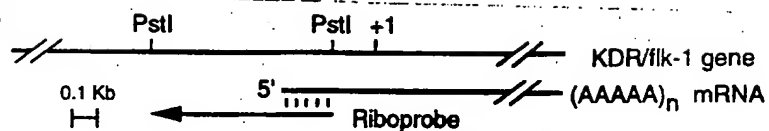
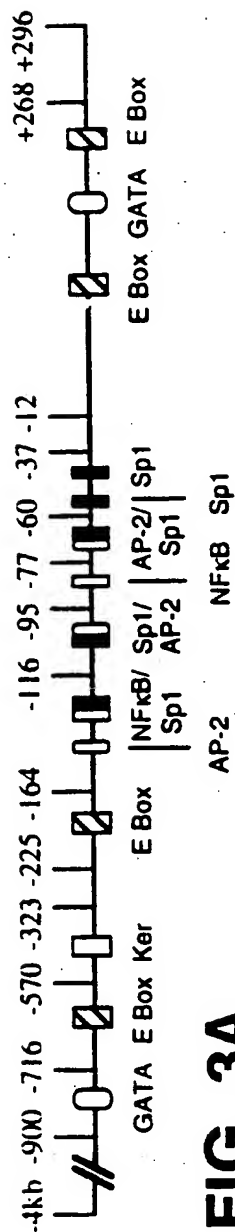


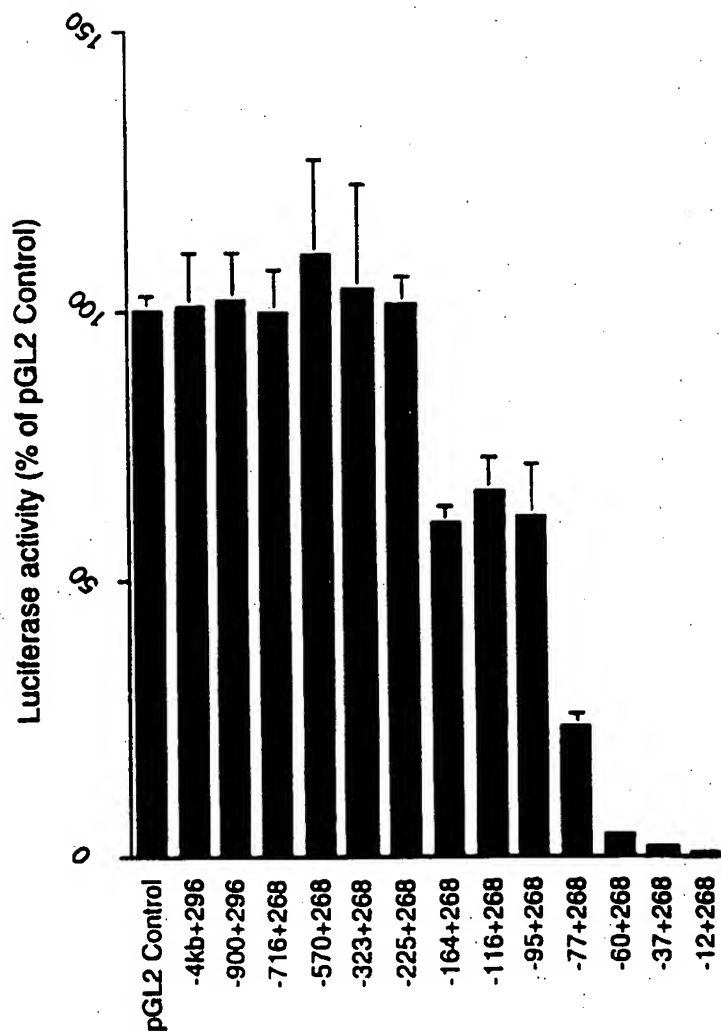
FIG. 2A

**FIG. 2B****FIG. 2C**

5/13

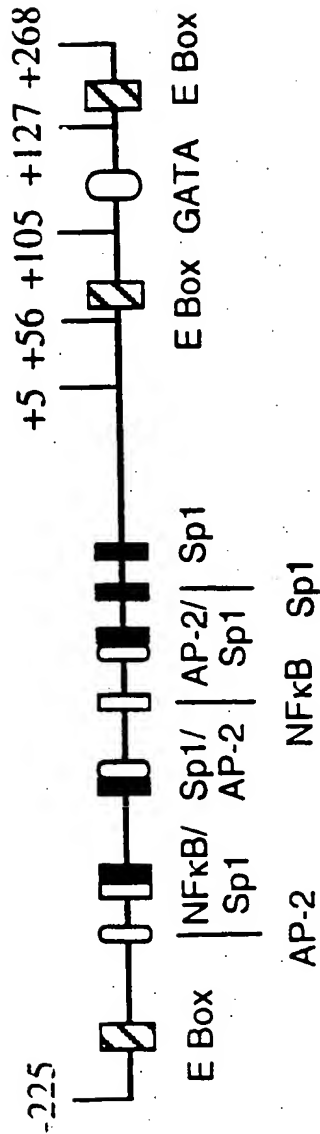


**FIG. 3A**



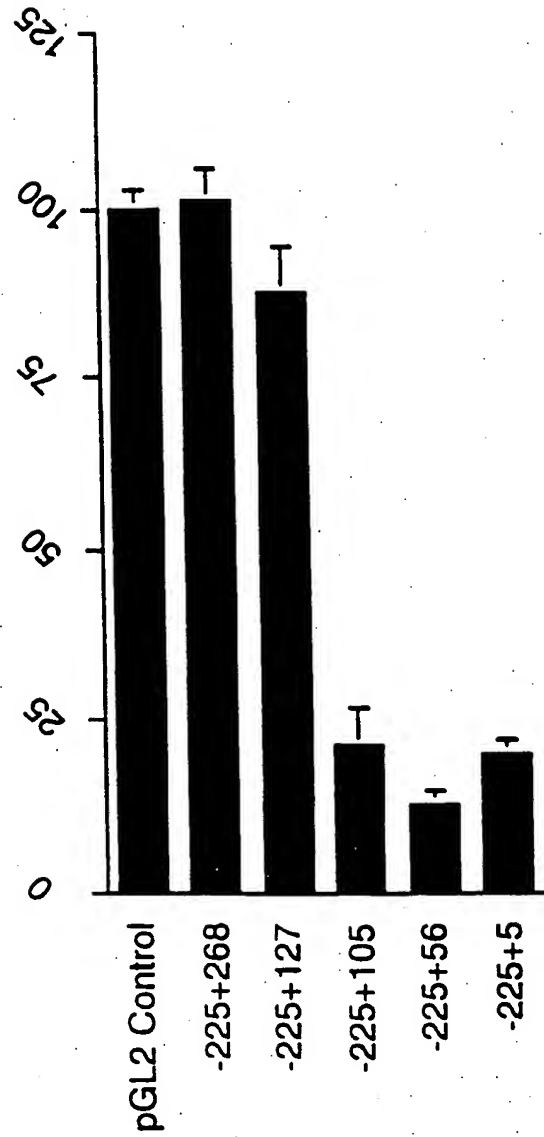
**FIG. 3B**

6/13



**FIG. 4A**

Luciferase activity (% of pGL2 Control)



**FIG. 4B**

7/13

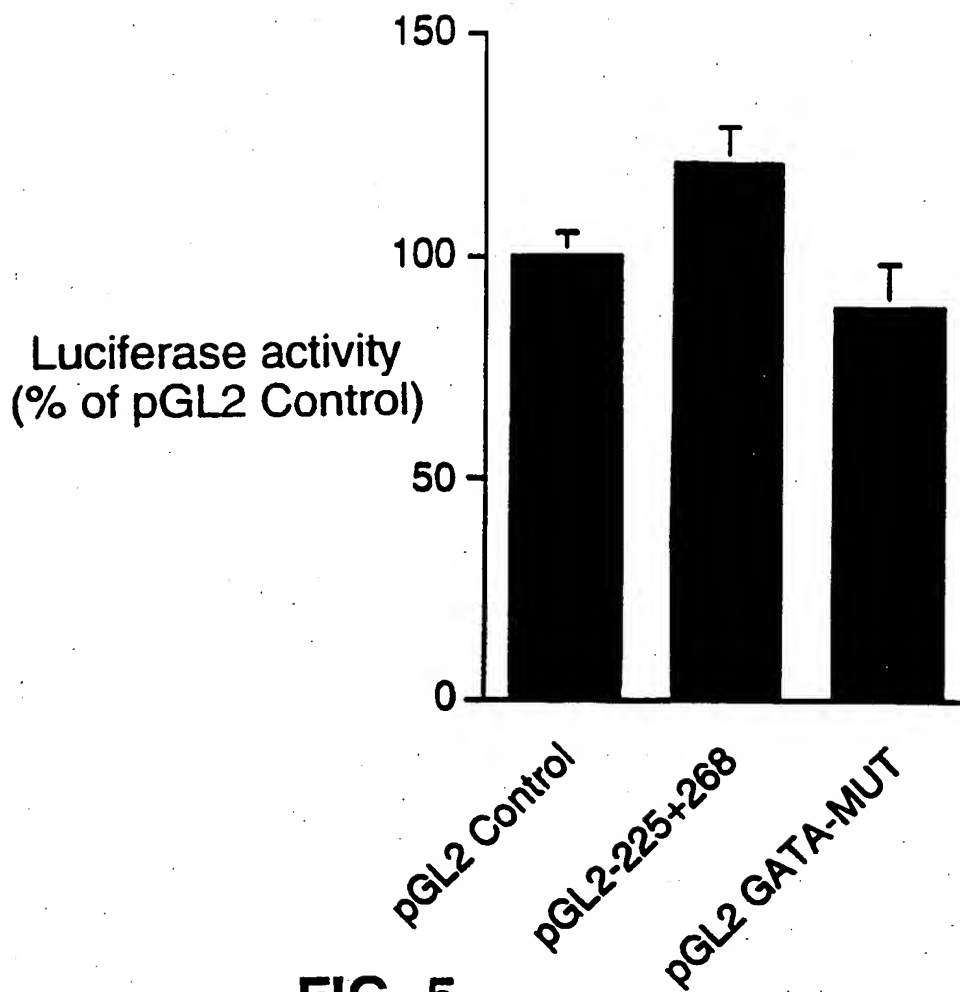


FIG. 5

8/13

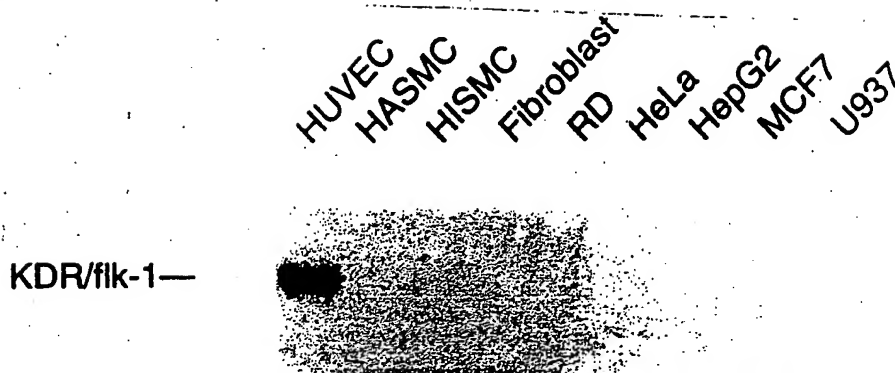
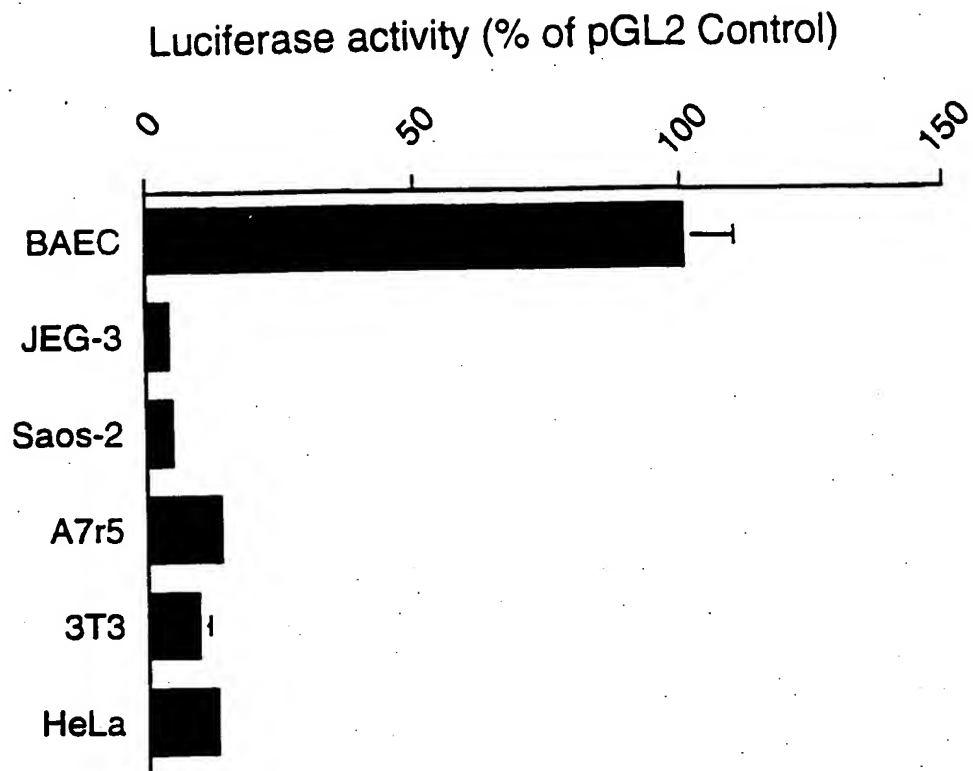


FIG. 6A



FIG. 6B

9/13

**FIG. 7**

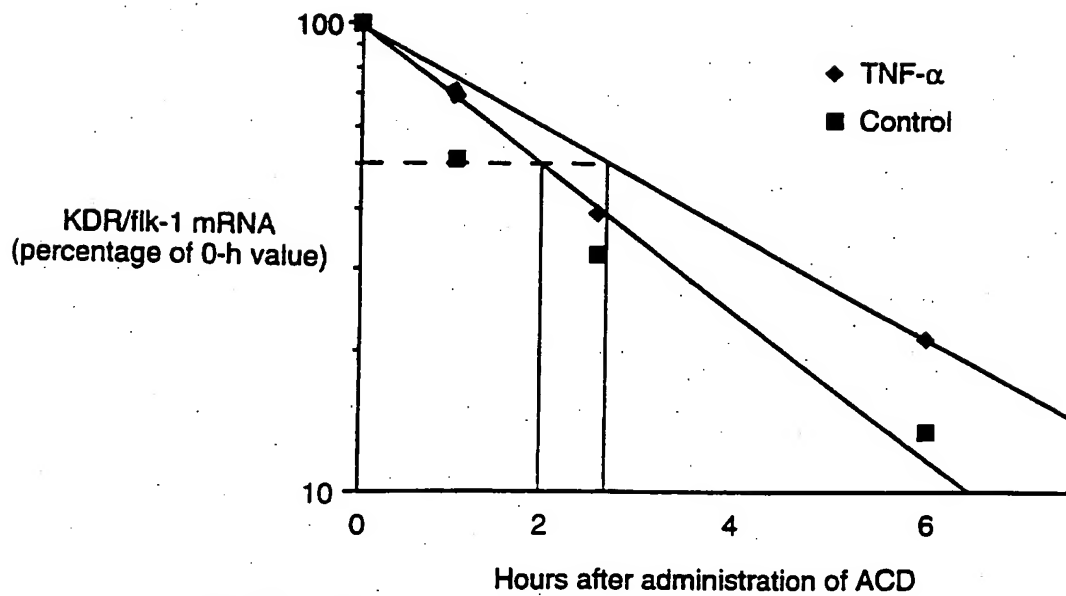
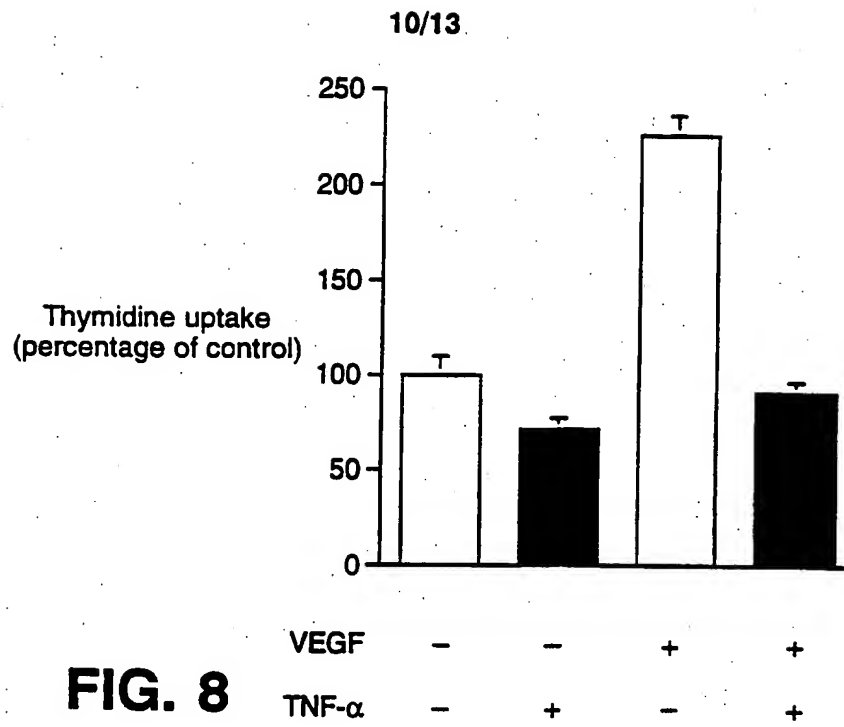
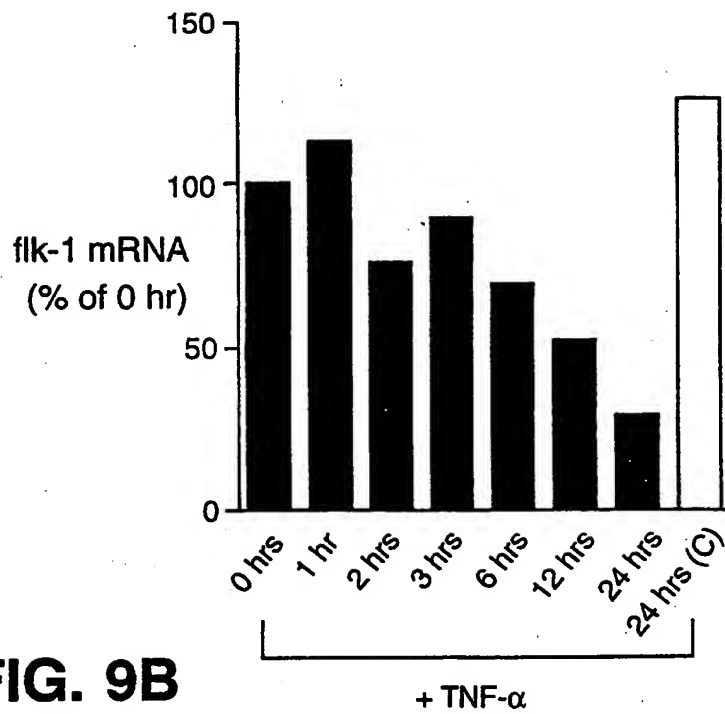
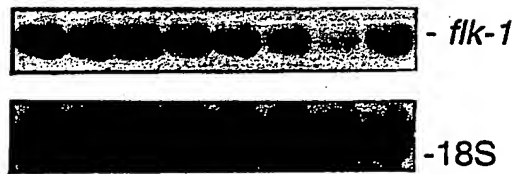


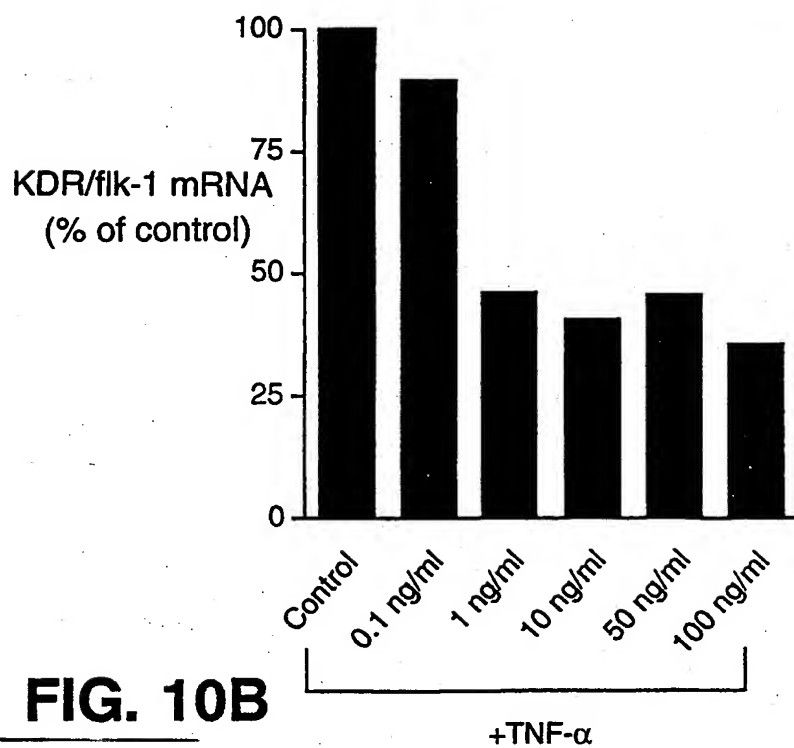
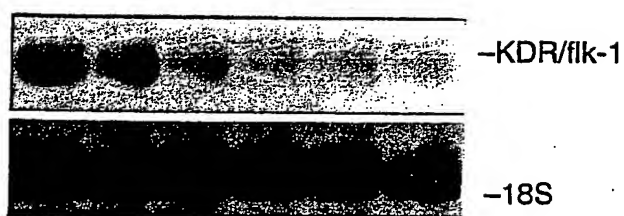
FIG. 11



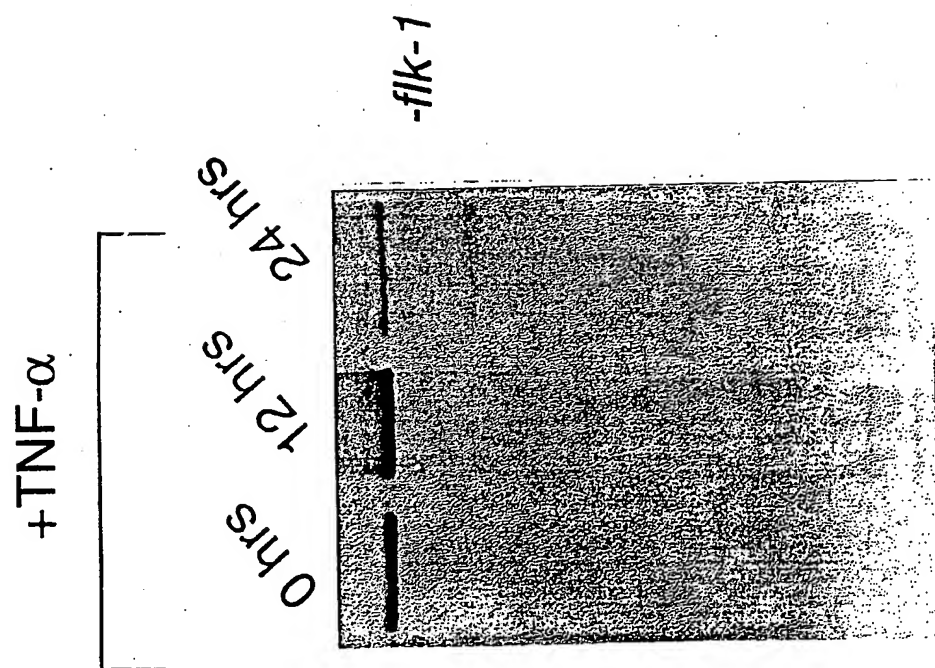
11/13

**FIG. 9A****FIG. 9B**

12/13

**FIG. 10A****FIG. 10B**

13/13



Immunoprecipitable  
flk-1 protein  
is decreased  
by TNF- $\alpha$

**FIG. 12**

## INTERNATIONAL SEARCH REPORT

International Application No  
PC./US 96/10725

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/71 C12N15/85 C12N5/10 A61K48/00  
C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X  A	<p>BREAST CANCER RESEARCH AND TREATMENT 36 (2). 1995. 139-155. ISSN: 0167-6806; XP000604149 KOLCH W ET AL: "Regulation of the expression of the VEGF -VPS and its receptors: Role in tumor angiogenesis."  see page 147, left-hand column, paragraph 2 - page 148, right-hand column, paragraph 1  --- -/--</p>	21-25

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

14 October 1996

Date of mailing of the international search report

30. 10. 96

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Authorized officer

Montero Lopez, B

## INTERNATIONAL SEARCH REPORT

International Application No

PC./US 96/10725

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO,A,94 11499 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 26 May 1994</p> <p>see page 3, line 1 - line 26  see page 8, line 7 - line 17  see page 23, line 3 - page 24, line 12  see page 30, line 18 - line 27  see page 34, line 11 - page 35, line 7</p> <p>---</p>	1-25
A	<p>WO,A,92 13063 (ONCOGENE SCIENCE, INC., USA) 6 August 1992</p> <p>see page 15, line 23 - page 16, line 6  see page 18, line 23 - page 21, line 4  see page 22, line 4 - line 21  see page 30, line 17 - page 32, line 15  see page 34, line 31 - page 37, line 12  see page 40, line 20 - page 41, line 24</p> <p>---</p>	1-25
A	<p>DEVELOPMENT,  vol. 121, no. 4, 1 January 1995,  pages 1089-1098, XP000604125  THORSTEN M. SCHLAEGER ET AL.: "Vascular  endothelial cell lineage-specific promoter  in transgenic mice"  see abstract  see page 1089, right-hand column,  paragraph 2  see page 1091, right-hand column,  paragraph 2 - page 1097, left-hand column,  paragraph 3</p> <p>---</p>	1-25
A	<p>WO,A,94 10202 (GENENTECH INC) 11 May 1994  see page 4, line 16 - page 6, line 2</p> <p>---</p>	21-25
P,X	<p>JOURNAL OF BIOLOGICAL CHEMISTRY,  vol. 270, no. 39, 29 September 1995, MD  US,  pages 23111-23118, XP002015662  CAM PATTERSON ET AL.: "Cloning and  functional analysis of the promoter for  KDR/flk-1, a receptor for vascular  endothelial growth factor"  see abstract  see page 23113, right-hand column, last  paragraph - page 23117, right-hand column,  paragraph 2; figure 1</p> <p>-----</p>	1-12

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 10725

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13, 14, 20, 23-25  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 13, 14, 20 and 23-25 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compounds.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC/US 96/10725

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9411499	26-05-94	AU-A- 5562794	08-06-94
		CA-A- 2149298	26-05-94
		CN-A- 1094445	02-11-94
		EP-A- 0669978	06-09-95
		JP-T- 8505763	25-06-96
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WO-A-9213063	06-08-92	AU-A- 1469292	27-08-92
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WO-A-9410202	11-05-94	AU-A- 2928992	24-05-94
		BG-A- 99605	29-02-96
		BR-A- 9207175	12-12-95
		EP-A- 0666868	16-08-95
		FI-A- 951987	26-04-95
		JP-T- 8502514	19-03-96
		NO-A- 951609	27-04-95
SK-A- 55195	09-08-95		
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